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## **PREAMBLE**

### **BACKGROUND**

1. Following the replacement of Test Guideline 401 on “Acute Oral Toxicity” with Test Guidelines 420, 423 and 425 in 2001, the WNT14 (Working Group of the National Co-ordinators of the Test Guidelines Programme) in 2002 found it timely to also develop alternative Test Guidelines for the “Acute Inhalation Toxicity Study” (TG 403), applying similar bio-statistical approaches. The WNT14 approved the proposal from the UK for development of a Test Guideline on “Acute Inhalation Toxicity – Fixed Concentration Procedure (FCP)” (draft TG 433) as a full replacement for TG 403. The draft was accompanied by a Guidance Document on acute inhalation toxicity testing that was initially called GD No. 39 (renamed 39A after the WNT16).
2. At the WNT16, a second proposal for an “Acute Inhalation Toxicity - Acute Toxic Class (ATC) Method” as a replacement method of TG 403 was presented by Germany and was approved by the WNT to be added to the rolling work-plan. The draft TG also came with a draft Guidance Document No. 39B.
3. The 1<sup>st</sup> version of TG 433 was circulated in November 2002 together with the draft guidance document and the 1<sup>st</sup> version of the draft TG 436 and GD39B was circulated in December 2004. A considerable amount of comments were received on both drafts.
4. Prior to the WNT17 meeting in April 2005, the US proposed updating the existing TG 403 so that it covers all regulatory requirements, in addition to addressing some reductions in the use of laboratory animals. The WNT17 meeting was unable to reach any decision on the way forward for these projects, especially considering that the new methods had been on the rolling work-plan as alternatives to TG 403 for several years.

### **THE MEETINGS**

5. The 1<sup>st</sup> Expert Consultation on Acute Inhalation Toxicity was held at the Federal Institute for Risk Assessment (BfR) in Berlin on 22-24 February 2006. The main purpose of the meeting was to revise the alternative draft Test Guidelines 433 and 436 in accordance to the comments received from member countries in previous circulation rounds and to develop a strategy how to finalise the draft TG’s as quickly as possible. In addition, the meeting also discussed the draft Guidance Documents No. 39A and 39B and how these drafts could be merged into one document, as decided by the WNT. Another important task of the meeting was to harmonize the alternative TG’s with the newly proposed revised TG 403. The meeting was also asked to discuss the scope of the Guidance Document and whether it should be constructed to encapsulate the essence of all three draft Test Guidelines.
6. The Berlin meeting successfully addressed a multitude of issues and in general the discussions were constructive and focused on solving problems and harmonizing the three draft TG’s to the extent possible. Since it was not possible to reach consensus at the meeting on the validation status and overall performance of draft TG’s 433 and 436, a Performance Assessment Group (PAG) that was lead by Germany was established to assess the performance of the methods by bio-statistical evaluations and simulations. Also a Guidance Document Drafting Group (GDDG) was established to merge the two draft GD’s.
7. The GDDG had a meeting in Berlin at BfR, in June 2006, to discuss the merging of the GD’s and to develop a work plan for future activities.
8. A 2<sup>nd</sup> Expert Consultation Meeting was held at US EPA in Washington DC 7-9 November 2006.

The main purpose of the meeting was to revise the existing TG 403 but issues on the alternative TG's 433 and 436, the PAG and the draft Guidance Document No.39 were addressed, and the documents were harmonised to the extent possible. The meeting agreed to preliminary adopt the PAG report, to add the C×T protocol to the draft TG403 and to establish a C×T Performance Assessment Group (C×T PAG) that would execute a similar bio-statistical analysis as was done by the PAG. A number of issues remained unresolved, *e.g.*, the use of evident toxicity.

9. The WNT19 generally agreed to the recommendations and the proposed work-plan by the meeting and endorsed the establishment of a C×T PAG. US EPA offered to host a final Expert Meeting in Washington DC in spring 2008 to resolve all remaining issues and come up with a final work plan for the finalisation of the acute inhalation projects. To speed up the process with the C×T PAG, a statistician co-ordinated this work and reported to the Expert Group well ahead of the planned meeting in 2008. The strategy was overall very well taken by the WNT. Regarding the alternative methods, it became evident at the meeting that the supportive validation material for the draft TG 433 may not be satisfactory, and that actual testing may have to be done to be able to establish the performance of the Fixed Concentration Procedure for acute inhalation.

10. At the WNT20, in April 2008, the UK announced they withdraw the proposal for the TG 433.

11. An Expert Consultation Meeting for revision of the Test Guidelines TG 403, 412 and 413 was held in De Bilt in the Netherlands on 18-19 June 2007. Regarding the TG 403, the goal of the meeting was to have a clear insight in regulatory needs of both methods (403 and C×T) and to agree on goals for the performance assessment of the C×T protocol and on a work plan, including time table and task assignment for the statistical analysis. The complete Performance Assessment Report of the C×T was available on 14 March 2008.

12. An Expert Consultation Meeting was held at US EPA in Washington DC 15-17 April 2007 for resolving the last remaining issues and to discuss the C×T Performance Assessment, in particular. The meeting approved the C×T Performance Assessment Report with some changes to the conclusions, as outlined in the C×T PAG Report. The meeting further made changes to the draft TGs and the GD, and after in-depth discussions and revisions of the PAG report, it was finally approved by the meeting.

## ACKNOWLEDGEMENT

13. Many experts have participated in the OECD meetings and working groups and the Secretariat would especially like to mention the experts that have been involved in the development and drafting of this document, the two Test Guidelines 403 and 436 and the two Performance assessment Reports for the TG 436 and the 403/C×T, including:

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## I. INTRODUCTION

### **Background**

1. In 1981, the OECD adopted Test Guideline 403 (TG 403)(1), which describes how to perform a traditional inhalation LC<sub>50</sub> study. Since then, TG 403 has been the only OECD Guideline for acute inhalation toxicity testing and the revised version comprise both a traditional LC<sub>50</sub> study as well as a Concentration versus time approach (C\*t).

2. OECD Test Guidelines are periodically reviewed in the light of scientific progress and animal welfare considerations. Development of an Inhalation Acute Toxic Class (ATC) method for inhalation was considered appropriate following the adoption of the revised oral ATC method (TG 423)(2)(3)(4)(5)(6)(7)(8)(9)(10) in December 2001 and the subsequent deletion of the traditional Acute Oral Test Guideline 401. The alternative Test Guideline No. 436 on “Acute Inhalation Toxicity – Acute Toxic Class Method uses serial steps and fixed target concentrations to rank test article toxicity for classification and labelling, according to the United Nations Globally Harmonized System (GHS) of Classification and Labelling of Chemicals (11).

3. In conjunction with the development of acute inhalation Test Guidelines, two other inhalation Test Guidelines have been updated:

- TG 412 - Repeated Dose Inhalation Toxicity: 28-day or 14-day Study (adopted 12th May 1981)(12)
- TG 413 - Subchronic Inhalation Toxicity: 90-day Study (adopted 12th May 1981)(13)

There are no chronic or carcinogenicity study Test Guidelines for the inhalation route. The following three TGs are under revision and may be used for any route of exposure including inhalation:

- TG 451 – Carcinogenicity Studies (adopted 12th May 1981)(14)
- TG 452 – Chronic Toxicity Studies (adopted 12th May 1981)(15)
- TG 453 – Combined Chronic Toxicity/Carcinogenicity Studies (adopted 12th May 1981)(16)

Although the main emphasis of GD 39 is acute studies, many of the principles of performing and evaluating acute inhalation toxicity studies also apply to repeated exposure studies. Thus, these TGs refer the reader to GD 39 for further guidance. Some guidance that specifically applies to repeated exposure studies can be found in this guidance document. It is important to note that TG 403, TG 436, TG 412, TG 413, TG 451, TG 452, and TG 453 are not specifically intended for the testing of nano-materials.

### **Global Regulations Utilising Acute Inhalation Data**

4. Alternative Guideline TG 436 is able to satisfy most regulatory needs providing a range estimate of LC<sub>50</sub> and GHS categorisation. TG 436 also uses considerably less animals than the revised TG 403. TG 436 cannot satisfy all regulatory and scientific needs, however, so the original 1981 TG 403 (1) was revised so that it uses fewer animals while incorporating scientific advancements. TG 403 provides maximum flexibility to characterize the entire range of the concentration-mortality relationship so that it can satisfy a variety of regulatory needs (17). For example, it can be used to derive Acute Exposure Threshold Levels (AETLs) for EU Emergency Planning and Land-Use Planning under the Seveso II Directive (96/82/EC), Acute Exposure Guideline Levels (AEGs) and Emergency Response Planning Guidelines (ERPGs) levels. These levels require lethal and sub-lethal endpoints over a variety of exposure durations. TG 403 can also satisfy the regulatory needs of the United Nations Committee of Experts on the

Transport of Dangerous Goods, the United States Department of Homeland Security, and the United States Department of Transportation which depend on  $LC_{50}$  values for toxic materials. It is also needed for addressing accidental exposures in submarines and spacecraft where there is no possibility for evacuation. Finally, it allows for studies to be performed in a manner which is timely, cost effective, and more humane to laboratory animals than the original TG 403.

5. For a glossary of terms see Appendix I.



## II. PURPOSE

6. The purpose of this document is to assist the regulated community and regulators in selecting the most appropriate acute inhalation TG so that particular data requirements can be met while reducing animal usage and suffering. This Guidance Document contains additional information on the conduct and interpretation of studies performed using TG 403 and TG 436.

7. TG 403 and TG 436 serve different regulatory needs and therefore have different levels of reliability/predictability. For some test articles, reliability may be significantly affected if it is difficult to achieve a specific stable target concentration, so elaborate pre-tests without animals may be needed to achieve a specific stable atmosphere concentration and particle size. It can also be difficult to achieve equivalent chamber concentrations and particle size distributions in the pre-test, sighting study, and main study. This can result in inconsistent responses in the animal studies. The test article concentration can determine which part(s) of the respiratory tract are most affected. For example, a low concentration of a highly water soluble gas or vapour may cause nasal irritation, but a high concentration may cause nasal irritation and also lung oedema (which may be fatal). Many test articles are generated in two phases (*e.g.*, equilibrium of liquid/solid aerosol and vapour). The method chosen to collect test atmospheres for the determination of actual concentrations should adequately collect all phases of the test article. As the ratio of these phases varies with concentration, so too does the site of deposition and toxicity. All portal-of-entry physiological responses (such as reflex bradypnea) may alter test article uptake due to hyper- or hypoventilation and metabolism. This can result in greater or lesser toxicity and an increase in inter-animal variability. It is not unusual for one sex to be more susceptible to a given test article. In inhalation studies with test articles acting at the portal-of-entry, male rodents tend to be more susceptible than female rodents, whereas females are commonly more susceptible in oral studies. The reason for this is that males, which have a higher metabolic rate than females, inhale more test article due to their higher body weights and ventilation relative to female rodents. In principle, the selection of TG 403 or TG 436 is driven by regulatory needs. However, the numbers of variables associated with inhalation tests show that a science-based selection is required to generate meaningful and robust data in order to achieve the desired objectives. These aspects are described in detail in Part II.

### III. DATA NEEDS

#### **Triggers of Inhalation Toxicity Testing**

8. Acute inhalation toxicity studies are the ideal means for characterizing acute inhalation hazards, but there are circumstances when requiring an inhalation toxicity study is not justified for humane, scientific, or practical reasons. Testing in GHS category 5 is generally discouraged and should only be considered when there is a strong likelihood that results of such a test would have direct relevance for protecting human health (see Appendix 2). As a rule, testing should be done unless there are compelling reasons for not testing. The decision to test or not test should be considered on a case-by-case basis using a weight-of-the-evidence approach. Acute inhalation testing is not required if the physical form of a test article, as it is marketed or used, precludes any human inhalation exposure (e.g., solid metal block, non-friable granules, composite elastic materials etc.).

9. While recognizing that the focus of acute inhalation studies is on the lethal endpoint and the toxicological mechanisms related to it, the repeated Guidelines enable the characterization of adverse effects following repeated daily inhalation exposure to a test article for at least 14 to 90 days (the latter covers approximately 10% of the lifespan of a rat). The data derived from these studies and especially the sub-chronic inhalation toxicity study can be used for quantitative risk assessments and for the selection of concentrations for chronic studies. The objective of these studies is to reveal target organs and sensitive non-lethal endpoints characterizing toxicity, including an analysis of the entire concentration-response/effect relationship. At the lower end this is the no-observed-adverse effect concentration (NOAEC), at the upper end this is the maximum tolerated concentration (MTC). The MTC should not affect longevity of the animal nor induce undue distress. The design of repeated inhalation studies preclude such effects to occur based on adequately designed pilot studies which are dealt with in the respective TG.

#### **Weight of Evidence**

10. Acute inhalation toxicity data are used to satisfy hazard classification and labelling requirements, to estimate the toxicity of mixtures, and to assess human health and environmental risk. The derivation of either a point estimate of the LC<sub>50</sub> value (using TG 403) or a range estimate of the LC<sub>50</sub> (using TG 436) generally meets the acute inhalation toxicity regulatory requirements for classification and labelling of industrial chemicals, consumer products, and many pesticide applications. Acute inhalation toxicity studies can also characterize hazards associated with end-user products (e.g., biocides used indoors, multipurpose spray cans, aerosolized cleansing agents, incense to repel insects etc.). Non-lethal endpoints representing the lower end of the concentration-response curve may be as useful as lethal endpoints. The data needs of the majority of OECD member countries can be met by testing at the *limit concentration* or the *maximum attainable concentration* (depending on the specific properties of the test article; see paragraphs 49-53). For highly volatile test articles, testing beyond the *limit concentration* may be necessary to meet specific regulatory needs. For animal welfare reasons, testing in excess of the limit concentration (i.e., in the GHS Class 5 ranges) is discouraged and should only be considered when there is a strong likelihood that results of such a test will have direct relevance for protecting human or animal health or the environment (11).

11. Repeated exposure inhalation toxicity data are used to satisfy hazard characterization requirements with focus on a no-observed-effect concentration (NOAEC). This value is achieved by empirical data characterizing the dose-response/effect relationship of relevant endpoints or benchmark analysis. Testing paradigms focus on the duplication of exposure regimens and atmospheres which have relevance to exposure patterns humans are likely to incur. Therefore, in these types of studies, the primary focus is not on the classification and labelling of substances/mixtures as being commercialized rather than on characterization of toxic mechanisms and exposure atmospheres causing to health hazards to repeatedly exposed humans. Part II of this guidance document will address these aspects in greater detail.

### **Definition of the Exposure Metric**

12. Acute inhalation toxicity studies should be based on mass concentrations to comply with the unit of analytical standard curve used for the analytical method. Thus, gas, vapour, and aerosol concentrations are expressed using a mass per volume metric, such as mg/L or mg/m<sup>3</sup>, where the mass concentration is related to the test article (and not to an arbitrarily selected analyte). This allows for a direct comparison of test articles regardless of their physical state.

### **Conversion of Units of Exposure Concentrations**

13. Although gases are always tested in mass units (*e.g.*, mg/L or mg/m<sup>3</sup>), mass units may be converted to volumetric gas units (parts per million, abbreviated “ppm” or “ppmV”) under standard conditions to comply with specific regulatory needs such as the GHS Classification System. These following algorithms may be used to perform conversions at 22°C and 101 kPa atmospheric pressure, the recommended conditions for animal testing (see paragraph 63):

$$\begin{array}{ll} \frac{\text{mg} / \text{L} \times 24,200}{MW} = \text{ppm} & \frac{\text{mg} / \text{m}^3 \times 24.20}{MW} = \text{ppm} \\ \frac{\text{ppm} \times MW}{24,200} = \text{mg/L} & \frac{\text{ppm} \times MW}{24.20} = \text{mg} / \text{m}^3 \end{array}$$

MW = Molecular weight

14. These algorithms imply that 1 mole of gas at the specified temperature and pressure occupies a defined molar volume of an ideal gas. Unlike mass units, volumetric gases units (*e.g.*, ppm) vary with temperature and pressure. The use of volumetric gas units is complicated by their inconsistent application. For example, gas concentrations are reported at 0°C by gas producers, 20°C by GHS, and 25°C by Patty’s Toxicology Handbook (18). To adjust for this disparity, calculators that perform mass↔volumetric unit conversions can be found on the internet, such as:

[<http://www.lenntech.com/calculators/converter-parts-per-million.htm>]

[<http://www.ccohs.ca/oshanswers/chemicals/convert.html>]

### **Optimizing the Performance of the Test**

15. All available information on the test article should be considered by the testing laboratory before conducting the study. A test article’s physical state affects classification because current GHS classification boundaries (11) are dissimilar for gases, vapours, and aerosols (see Appendix II). Key information may include the identity and chemical structure of the test article, its composition (for mixtures) and physico-chemical properties (*e.g.*, vapour pressure), the results of any relevant toxicity tests on the test article, available (Q)SAR data and toxicological data on structurally related test articles, and the anticipated use(s) of the test article. Knowledge of dustiness and particle size for solid test articles will allow for selection of the ideal testing approach and starting concentration that will enhance respirability (*e.g.*, through the use of micronization). Factors that enhance potential human exposure due to physico-chemical properties or specific use pattern need to be considered. In this context, testing in GHS Class 5 should only be considered when there is a strong likelihood that the data gained will have direct relevance

for protecting human or animal health. If inhalation is not a likely route of exposure for a given test article, then reasons for not testing by this route should be provided. This Guideline gives preference to the nose-only mode of exposure, but it does not exclude other modes of exposure. While nose-only is the default mode of exposure, special objectives of the study may be better achieved by using the whole-body mode of exposure. The use of non-default modes of exposure should be based on the focus of the study and should be justified in the study report.

### **Data Bridging**

16. Some national and international regulatory systems estimate the toxicity of a mixture (formulation) using weighted averages of the LC<sub>50</sub> point estimates for each component when actual data on the mixture are not available. The resulting calculated toxicity values are then used for hazard classification. Especially for mixtures, available information should be utilized as “bridging principles” which enable suppliers to derive a sound classification of mixtures with a minimum of experimental animals. A concentration-response curve is sometimes needed for extrapolation and reliable identification of hazard and risk posed by mixtures. At present, agreed approaches for estimating the toxicity of mixtures using range data are only accepted in the EU and in some other countries. However, the GHS has recently agreed that mixtures can be classified using either point or range estimates of the LC<sub>50</sub> of each component (11). However, inhalation testing may be required if the percentages of components in the test article differ appreciably following aerosolization or evaporation due to dissimilar physico-chemical properties. Therefore, the test principles detailed in ‘Part I: Test Method Selection’ should be observed carefully.

17. When testing simple mixtures (*e.g.*, agrochemical preparations) of well characterized components, the Finney equation as defined by WHO (19) may be used to estimate an LC<sub>50</sub> (see equation below), provided these components produce additive acute toxicity and have parallel regression lines of probit against log doses. The estimated LC<sub>50</sub> can then be verified or refuted by performing a TG 436 study or this information can be used as a starting point for testing in place of a sighting study.

$$\frac{1}{LC_{50}} = \left( \frac{\% \text{ ingredient}_1}{LC_{50_1}} \right) + \left( \frac{\% \text{ ingredient}_2}{LC_{50_2}} \right) + \dots + \left( \frac{\% \text{ ingredient}_n}{LC_{50_n}} \right)$$

18. Alternatively, the acute toxicity estimate (ATE) of the GHS (chapter 3.1.3.) “Classification Criteria for Mixtures” can be applied. For mixtures, it is necessary to obtain or derive information that allows the criteria to be applied to the mixture (of different particle sizes) for the purpose of classification. The following equation is used to derive an ATE value:

$$\frac{100}{ATE_{mix}} = \sum_n \frac{C_i}{ATE_i}$$

where:

C<sub>i</sub> = concentration of the ingredient i of n ingredients, and i is running from 1 to n

ATE<sub>i</sub> = Acute Toxicity Estimate of ingredient i

19. Any conversion from experimentally obtained acute toxicity range values (such as ranges obtained by using TG 436) to acute toxicity point estimates should be based on the GHS (3.1.3., Table 3.1.2)(see Appendix II).

20. Before existing inhalation toxicity study data can be used for bridging purposes, the quality of the exposure data and the consistency of animal data should be assessed. Common pitfalls include

inappropriate methodologies to generate respirable aerosols or characterize exposure atmospheres. When data from several acute inhalation toxicity studies are available, scientific judgment should be used in selecting the study that was best performed and characterized.

### **Feasibility of Testing Mixtures**

21. Because a limit test (described below and in Appendices II and III) is commonly used when testing mixtures (end-use products), preference should be given to using TG 436 as illustrated in Figure 1.

### **Evidence from Humans**

22. For classification purposes, reliable epidemiological data and experience on the effects of substances on humans (*e.g.*, occupational data, data from accident data bases) should be considered in the evaluation of human health hazards. Human data that are reliable and of good quality will generally have precedence over other data. Human data will not necessarily supersede well-conducted animal studies, but rather the human and animal studies should both be assessed for their quality, the robustness of their data, and the impact of potentially confounding factors. Human testing solely for hazard identification purposes is not acceptable.

### **Applicability of Test Methods for Testing Pharmaceuticals**

23. Acute inhalation toxicity testing by TG 403/436 may not be relevant for pharmaceuticals. The International Committee on Harmonization (ICH) specifies pharmaceutical methods. Study designs for special purpose-driven studies differ from current OECD acute toxicity Guidelines, which are primarily designed for comparative evaluation and assessment of acute (lethal) toxic potency. These studies typically characterize pharmaceuticals with very low toxicity and thus may require test concentrations above the respective *limit concentration* detailed in Appendix II.

#### IV. COMPARISON OF GUIDELINES 403 AND 436

##### **Outline of the Exposure Methodology**

24. Acute inhalation toxicity is the total of adverse effects caused by a test article following a single, uninterrupted exposure of non-fasted healthy young adult animals by inhalation over a short period of time (less than 24 hours) to an adequately generated and characterized test article atmosphere. The total of adverse effects is best described by cumulative mortality. A fixed duration exposure of 4 hours is generally recommended but shorter or longer exposure durations may be appropriate to meet specific objectives. The limiting duration for nose-only exposure for rats is generally 6 hours. If other species are used, shorter exposure durations may be indicated to prevent undue species-specific distress. When using species other than rats, justification for exposure durations other than 4 hours should be provided. An observation period of at least 14 days after exposure, recording of body weights at regular intervals, and the necropsy of all animals is recommended. Technological details are addressed in Part II of this document. Some authorities prefer that end-use products sold to the public should be tested in a way that reflects most closely the anticipated exposure pattern. Also the selection of a vehicle should be based on these considerations. If acute inhalation testing of the test article was omitted due to a lack of likelihood of exposure (see paragraph 15) then testing of the mixture becomes mandatory if its content in the mixture exceeds 0.1%. The preferred mode of exposure is nose-only. This particular exposure mode allows for the testing of multiple exposure durations using the same exposure atmosphere in order to obtain a range of concentration x time (C×T) relationships (20). While nose-only is the default mode of exposure, special objectives of the study may be better achieved by using the whole-body mode of exposure.

25. This Guidance Document primarily describes studies performed in commonly used rodent species (generally the rat), but it may also be adapted for studies in non-rodent species. Animals should be randomly assigned to the experimental groups. Most animal suppliers do not indicate litter mates so the Guidelines do not call for randomizing animals from a single litter across exposure groups. Females should be nulliparous and non-pregnant. On the exposure day, animals should be young adults 8 to 12 weeks of age, and body weights for each sex should be within  $\pm 20\%$  of the mean weight of all previously exposed animals at the same time point. As the mean weight increases, respiratory minute volume will also increase, though not in a proportional manner.

26. The determination of acute inhalation toxicity is usually an initial step in the assessment and evaluation of the toxic characteristics of an inhaled test article whether it is a gas, vapour, or aerosol (*e.g.*, dust, mist, smoke, fume, fog, or smog). It provides information on health hazards likely to arise from short-term exposure by the inhalation route. An evaluation of acute toxicity data should include the relationship, if any, between the animals' exposure to a specific test article chamber concentration and the incidence and severity of all abnormalities, including behavioural and clinical effects, the reversibility of observed effects, gross lesions, body weight changes, effects on mortality, and any other toxic effects. Elaborate technical measures are often taken to maximize exposure to the entire respiratory tract, and to assure temporal and spatial stability of exposure concentrations.

27. Test atmospheres in inhalation chambers may consist of a mixture of different phases (*e.g.*, vapour, liquid aerosol, or the equilibrium thereof). Because of the need to generate respirable particles, the fraction of airborne particles generated from a mixture of polydisperse particles may not mirror a test article's aerosol characteristics under conditions of use. These aspects should be considered when judging the toxicological significance of findings from acute inhalation toxicity tests.

28. Acute inhalation toxicology testing Guidelines and available technologies have improved significantly over time, both in terms of well-defined animal exposure and test atmosphere characterization. Especially for short-term inhalation studies, exposure paradigms have shifted from whole-body to nose-only modes with novel procedures that minimize the re-breathing of atmospheres, attain faster inhalation chamber concentration equilibrium, and optimize the uniformity (*i.e.*, degree of dynamic

mixing) of flows within an inhalation chamber. The availability of computer-supported real-time monitoring devices and increased analytical sensitivity allows for better attainment of a uniform, spatial dispersion and temporal stability of test articles in an inhalation chamber. This dependence on available technologies when exposing experimental animals is unique to inhalation toxicology.

29. Experimental animals may either be exposed whole-body (horizontal and vertical flow type chambers, small, medium, and large size chambers with laminar, circular or turbulent flow arrangements to enhance the homogeneity of inhalation chamber concentrations) or nose-only (in mixed-flow, directed-flow, or flow-past inhalation chambers) with positive, negative, or zero flow gradients across the animals' breathing zones. Each arrangement may require specific considerations which are partially addressed in this document. Historical data should demonstrate that horizontal/vertical concentration gradients in the inhalation chamber and bias airflows which dilute breathing zone atmospheres do not occur to any appreciable extent. The following should be considered when choosing an inhalation chamber: 1) reactivity of test article with humidity and/or ammonia, 2) temporal stability of test atmosphere, e.g. minimization of particle growth and coagulation/aggregation, 3) prevention of re-breathing of test atmospheres, and 4) measurements and/or collection of biological specimens during the course of exposure (20).

30. The characterization of solid and liquid aerosols in inhalation chambers frequently requires that an aerosol sample be conveyed to a measurement or collection device. This is accomplished by withdrawing a sample from an inhalation chamber such that the sample is representative of the aerosol in the animals' breathing zone and not affected by the sampling process. Many mechanisms that affect representative sampling depend on aerosol particle size and airflow rates. A given sampling system may exhibit representative sampling over a specific particle size range but may not be able to characterize particles larger or smaller than that range. One objective of this Guidance Document is to clearly specify the importance of particle size and to describe how to minimize sampling errors. This means that "isokinetic" sampling strategies to preserve chamber aerosol characteristics need to be considered so that all phases and particle size fractions of a specific analyte are collected with high efficiency from the animals' breathing zone in order to obtain similar material mass balances from different procedures.

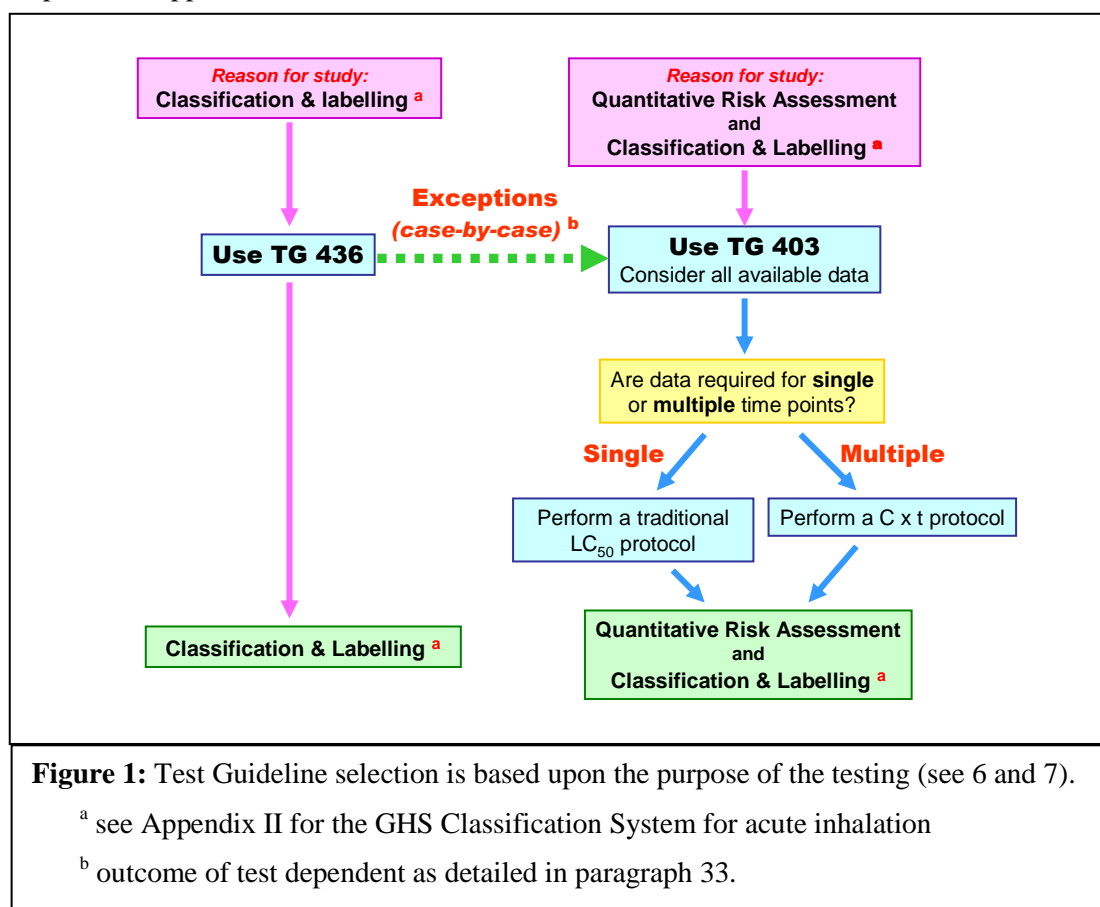
## V. TEST GUIDELINE SELECTION

### Prioritization of Test Guideline

31. When range estimates are needed for the sole purpose of classification and labelling, TG 436 should be given preference because this alternative method provides significant reductions in the number of animals used (for details see Appendix III). Conversely, the focus of TG 403 is on the analysis of the entire concentration-response relationship ranging from non-lethal to lethal outcomes in order to derive a median lethal concentration (*e.g.*,  $LC_{50}$ ), non-lethal threshold concentration (*e.g.*,  $LC_{01}$ ), and slope. The higher level of information provided by the two protocols in TG 403 should be judiciously counterbalanced by the number of animals used to achieve this objective. All TGs include a requirement to follow the OECD Guidance Document on Humane Endpoints (see paragraphs 39-41) which should reduce the overall suffering of animals used in acute toxicity studies and provide useful data for human risk characterization.

### Test Guideline Selection

32. The decision tree shown in Fig. 1 depicts when it is appropriate to use TG 403 or TG 436. The selection of a testing approach is based upon a test article's specific data requirements. TG 436 should be given preference if it is able to satisfy regulatory or scientific needs. Whenever the objective of the test is to perform a *limit test* or a test at the *maximum attainable concentration* with an anticipated non-lethal outcome, TG 436 should be used. If there is a regulatory or scientific requirement for an assessment of the concentration response relationship, with or without a detailed analysis of the C×T relationship, then TG 403 is the preferred approach.





33. A study director or principal investigator should consider the following scenarios when selecting a Test Guideline for a given test article.

#### **Existing Evidence**

- An attempt should be made to predict the outcome of a test by read-across/bridging/QSAR procedures, especially for mixtures with components of known toxicity.
- If such a prediction can be made with high confidence, testing should start with one single point estimate (*e.g.*, an estimated LC<sub>50</sub> or a limit concentration).
- If the assumption is refuted, the test result can be used to define the starting point for a TG 436 study.

#### **Regulatory needs**

- Regulatory requirements should be consulted to determine if results obtained from a TG 436 study will be adequate.
- A TG 403 study should be performed if there is a regulatory/consumer protection need for a lethality point estimate (*e.g.*, an LC<sub>50</sub>, LC<sub>10</sub>, etc.), a concentration-response analysis, and/or sex susceptibility quantification.

#### **Test articles that are anticipated to be highly toxic**

- Some highly toxic test articles may pose a unique health hazard. If a test article is classified as Class 1 or 2 in a TG 436 study, or if there is information that suggests it will likely be classified as Class 1 or 2, then consideration should be given to performing a TG 403 study so its toxicity can be further characterized.
- This may also apply to highly volatile test articles.

#### **Test articles that are severely irritating or corrosive**

- Test articles that are irritating or corrosive should always be tested using TG 403 because it provides the study director or principal investigator with control over the selection of target concentrations. Dilutions of corrosive test articles may be tested at exposure concentrations sufficient to extend the concentration-response curve to levels that reach the objective of the test and thus serve regulatory and scientific needs.

#### **Technical problems**

- Technical problems may be encountered that make it impractical to perform a TG 436 study with its fixed concentrations. For example, if it is difficult to achieve the target chamber atmosphere concentration during pre-testing (before animals are exposed), then a TG 403 study should be performed. A TG 403 study is less affected by deviations from target concentrations because statistical analysis considers whatever actual concentrations were achieved.

#### **Future changes in the GHS category bands**

- Changes to GHS category bands in the future will require a reassessment of biometrical performance (target) of TG 436 studies. This cannot occur with TG 403 studies because the concentrations tested are not fixed to GHS cut-off values.

#### **TG 403 Studies - Traditional protocol or C×T protocol?**

- If a fixed point estimate of lethality is needed (*e.g.*, a 4 hour LC<sub>50</sub>), a Traditional LC<sub>50</sub> protocol should be performed.
- If an estimate of the effect of time on concentration is needed, a C×T protocol should be performed.
- If information is needed on LC<sub>10</sub> or LC<sub>01</sub> values, a C×T protocol will provide better estimates than a Traditional LC<sub>50</sub> protocol (20).
- It is the responsibility of the investigator to determine whether the desired objectives are better achieved with the Traditional LC<sub>50</sub> protocol or the C×T protocol.

#### **Sighting Studies**

34. **TG 403:** A sighting study is used to estimate test article potency, to identify sex differences in susceptibility, and to assist in selecting exposure concentration levels for the main study. A sighting study using up to three animals/sex/concentration (for details see Appendix III) may be needed to choose an appropriate starting concentration for the main study and to minimize the number of animals used. It may be necessary to use three animals/sex to establish a sex difference. The feasibility of generating adequate test atmospheres should be assessed during technical pre-tests without animals. It is generally not necessary to perform a sighting study if mortality data are available from a TG 436 study. When selecting the initial target concentration in a TG 403 study, the study director should consider the mortality patterns observed in any available TG 436 studies for both sexes and for all concentrations tested.

35. **TG 436:** This Guideline does not call for a sighting study.

#### **Main Studies**

36. **TG 403:** This Guideline allows a study director or principal investigator to choose between two types of studies depending on regulatory and scientific needs—a traditional LC<sub>50</sub> study or a C×T study. In a traditional LC<sub>50</sub> study, 5 rats/sex/concentration are exposed in a stepwise procedure. The lowest selected concentration is expected to produce some signs of toxicity but little or no mortality, and the highest concentration is expected to be lethal to most of the animals. The C×T study tests multiple concentrations and exposure durations. Each exposure atmosphere can be used to obtain a range of concentration x time (C×T) relationships by periodically placing and removing animals in a nose-only chamber for predetermined durations. For both study designs, testing should be performed in a single sex if one is known to be more susceptible. GHS toxicity classification with this Guideline is based on mortality and the derivation of a statistically derived median lethal concentration (*e.g.*, LC<sub>50</sub>), confidence interval, and slope. Other regulatory requirements may require estimation of additional lethal toxicity indices (*e.g.*, LC<sub>01</sub>, LC<sub>10</sub>, etc.).

37. **TG 436:** Pre-specified fixed concentrations are used in the main study. Groups of 3 animals/sex (or 6 animals of the susceptible sex) are simultaneously exposed in a stepwise manner, with the initial concentration being selected to produce mortality in some animals. Depending on the presence or absence of mortality, further groups of animals may be exposed at higher or lower fixed concentrations as set out in Appendix II of TG 436 until it is possible unequivocally to classify the test article. Because accuracy in

achieving each target concentration is paramount to assure accurate classification and labelling, a technical pre-test without animals is mandatory. Although most studies will be 4 hours in duration, other exposure durations may be used to serve specific regulatory purposes.

### **Information Provided by Each Test Guideline**

38. The results of tests conducted according to TG 403 and TG 436 allow a test article to be classified according to all the systems in current use, including the GHS Classification System. In addition:

- TG 436 provides a range estimate of the LC<sub>50</sub> instead of a point estimate. The ranges, as defined by GHS classification cut-off values, are different for each physical state of the test article under test conditions (gas, vapour, aerosol) (see Appendix II).
- The Traditional LC<sub>50</sub> protocol in TG 403 provides a point estimate of the LC<sub>50</sub> value with confidence intervals when at least 3 data points (concentrations levels) are available with finite probabilities of mortality. In case there are only two data points with mortality close to 0% and 100% available (*i.e.*, a very steep concentration-mortality relationship), they can be used to estimate an “approximate LC<sub>50</sub>.” The approximate LC<sub>50</sub> is defined as the geometric mean from these mortalities.
- The C×T protocol in TG 403 yields a matrix of data points for a range of concentrations and durations that can yield point estimates for a variety of durations. The C×T protocol works very well in case of steep concentration-mortality relationships because a C×T study relies on both concentrations and durations rather than concentrations alone.

### **Animal Welfare Considerations**

39. Ethical concern for the welfare of experimental animals includes the alleviation of stress and suffering. In addition to allowing for classification and labelling, acute inhalation toxicity studies may provide important information regarding potential hazards that may be associated with the use of consumer products (*e.g.*, indoor biocides, multipurpose spray cans, aerosolized cleansing agents, insect repellent incense, etc.). To this end, the non-lethal endpoints at the lower end of the concentration-response curve might be as useful as lethal endpoints. Whenever this objective can be achieved by using alternative test methods, which use fewer animals, this approach should be taken.

40. The revised TG 403 and TG 436 use fewer animals than the 1981 version of TG 403 and they all contain a requirement to follow the OECD Guidance Document No. 19 on Humane Endpoints (22), which should reduce the overall suffering of animals used in acute inhalation toxicity testing. TG 403 uses a sighting study to minimize the number of animals needed. TG 436 has stopping rules which limit the number of animals used in a test.

41. Animals showing severe and enduring signs of distress and pain should be humanely killed as described in OECD Guidance Document no. 19 (22). When exposing animals to a test article with corrosive or strong irritant properties, the targeted concentrations should be low enough to not cause marked pain and distress. Test articles that are eye/skin irritants may also be respiratory tract irritants. Due to markedly different methodological approaches, the results from eye/skin corrosivity tests cannot readily be translated to actual inhalation exposure concentrations delivered over a specified time period. Therefore, corrosive test articles should be assessed and tested following expert judgment on a case-by-case basis.

### **Limitations of Particular Approaches**

42. Validations against actual data and statistical simulations identified areas where TG 436 may have outcomes which result in a more or less stringent classification than that based on the “true” LC<sub>50</sub> value (as obtained by TG 403) due to the fact that the ranges are defined by GHS cut-off values.

Comparative statistical analysis (17) demonstrates that a method that provides a range estimate of the  $LC_{50}$  is likely to perform poorly for chemicals with shallow concentration-response slopes. Some test substances cause delayed deaths (5 days or more after exposure to the test article) which may have an impact on the practicality of conducting a study using TG 436. The finding of a delayed death may require additional lower concentration levels to be used or a study to be repeated. The classification boundaries of GHS are not equidistant across classification classes and are inconsistent between gases, vapours, and aerosols (dusts and mists). Thus the required reliability/precision changes from one class to another. Therefore, scientific judgment is needed to decide which of the TG's will best achieve the objective of the test.

43. Unlike the TG 403 approach where point values are estimated by established statistical procedures, TG 436 studies require a greater measure of accuracy and consistency in chamber atmosphere because they solely depend on the outcome at the targeted exposure cut-off. This is why a technical pre-test without animals is required for TG 436 studies. Although this may be time-consuming and result in a protraction of the study, it is necessary to assure that the target concentration and particle size (for aerosols) are attained. Appendix III details the variation that should not be exceeded for the targeted point estimates used in TG 436 studies. A protracted study may both increase the day-to-day variability of testing and affect the body weights of pre-assigned animals. These factors are of less concern when using TG 403 because the incremental steps and the associated changes in the physical characteristics of exposure atmospheres are commonly smaller than the cut-off limits of classification boundaries (see Appendix II) and because statistical analysis uses the actual concentrations. Nevertheless, technical pre-tests are recommended when performing a TG 403 study to maximize the likelihood of successful tests.

44. Literature surveys of systemically acting test articles show that there is usually little difference in susceptibility between the sexes in oral and dermal acute toxicity studies (23). However, in those cases where differences were observed, females were slightly more susceptible due to the lower detoxification capacity of females compared to males. Equivalent dosing in both sexes is easily achieved via the oral and dermal routes, but not by the inhalation route. Consequently, sex susceptibility is based on inhaled dose as well as metabolism. For example, males in acute inhalation studies typically have greater body weights and correspondingly higher ventilation leading to a higher inhaled dose as compared to females of the same age. Differences in the toxification/detoxification capacities may be more pronounced in inhalation studies than in bolus administration procedures (*e.g.*, gavage studies) when the rate of delivery of the test article does not overload the available toxification/detoxification capacities. The inter-animal "dosing variability" due to changes in ventilation is potentially higher in inhalation studies than in non-inhalation studies. Consequently, single sex studies are justified only if distinctive sex differences are observed. The susceptibility of a specific sex depends heavily upon the chemical class and the associated mode of action. When both sexes are examined, simultaneous testing of both sexes is recommended because it is difficult to exactly reproduce identical exposure atmospheres when testing is sequential, especially with aerosols.

## VI. CONDUCT OF STUDIES

### PRINCIPLE OF THE TEST

#### **Technical Pre-Tests**

45. The feasibility of generating a targeted atmosphere should be determined in a pre-test without animals. Pre-tests are mandatory for TG 436 and recommended for TG 403 to prevent useless animal exposures. Each test article may pose unique physical challenges and/or require vehicle systems to generate and characterize the test atmosphere. This pre-test can show that a stable inhalation chamber atmosphere can be generated at the target concentration and particle size (for aerosols; see below). Collection efficiency and sampling error of equipment used to characterize an atmosphere should be ascertained. The equipment used to sample chamber atmospheres (*e.g.*, flow-limited critical orifices, gas meters, or flow controllers) should be regularly calibrated. Evaporated constituents from the test atmosphere or the collection medium (*e.g.*, glass bubblers containing volatile solvents) should not interfere with the precise determination of the sampled volume. Ideally, the comparison of results obtained from different equipment should identify technical inconsistencies and verify that sampling errors do not occur to any appreciable extent.

46. In the case of highly reactive materials (reaction potential with moisture, oxygen etc.) the test atmosphere should be fully characterised and its relevance to the potential human exposure situation should be considered. For example, it may be acceptable to expose animals to degradation products in air as this will represent the actual overall hazard to humans in the workplace/environment. Controlled dried air is always used for generation during inhalation studies, and normally the moisture content is low enough not to result in delivery issues. Diluent air, if used, is dried to lesser degree and can also be humidified to a level consistent with ambient to emulate the hazard environment. In repeated inhalation studies using generally markedly lower concentrations than in acute inhalation studies the stability and homogeneity of atmospheres needs to be verified by appropriate analytical methodologies.

#### **Control Group**

47. A concurrent negative (air) control group is not necessary for acute studies. When a vehicle other than water is used to assist in generating the test atmosphere, a vehicle control group should be used when historical inhalation toxicity data are not available. If a toxicity study of a test article formulated in a vehicle reveals no toxicity, it follows that the vehicle is also non-toxic at the concentration tested so there is no need for a vehicle control. To allow for statistical comparisons of non-lethal endpoints, adequate historical data from a similarly exposed control group may help in distinguishing between specific effects caused by the test article and non-specific effects associated with the method of exposure.

#### **Vehicle**

48. If the targeted concentration cannot be attained using the undiluted test article, a vehicle should be used. The selection of the vehicle should be based on previous experience, the pattern of use (*e.g.*, water for pesticide formulations that are sprayed in water) or physical restraints (solubility and stability of test article, particle size). A vehicle may also be considered to enhance the dustiness of solid test articles (powders). The kind and concentration of vehicle should not interfere with the outcome of the study with regard to the airborne test article's analytical stability or toxicity. Ideally, the vehicle selected should be non-toxic with water being given first preference. When a vehicle other than water is used, a vehicle control group should only be used when historical inhalation toxicity data are not available. If a concurrent vehicle control is to be avoided, historical data should show that the vehicle does not interfere with the outcome of the study.

## **Limit Test**

49. The limit test is primarily used when the test article is known to be virtually non-toxic, *i.e.*, eliciting a toxic response only above the regulatory limit concentration. Limit tests evaluate the targeted limit concentration or, if technically not achievable due to the test article's physicochemical nature, the maximum attainable concentration. For gases and vapours, there is no need for further testing if less than 50% lethality occurs at the limit concentration or the maximum attainable concentration (in case the actual limit concentrations is in the range of the vapour saturation concentration). For aerosols, the MMAD of the test atmosphere should be considered if no deaths occur at the limit concentration or the maximum attainable concentration. If the MMAD significantly exceeds 4 µm, further efforts should be employed to reduce the test article's MMAD. If the test atmosphere achieves the recommended MMAD standard of 1-4 µm and less than 50% lethality occurs at the limit concentration or the maximum attainable concentration, no further testing is necessary.

50. The selection of limit concentrations usually depends on regulatory requirements. When the GHS Classification System is used, the limit concentrations for gases, vapours, and aerosols are 20,000 ppm, 20 mg/L, and 5 mg/L, respectively (see Appendix II). The GHS limit concentrations are used in TG 436 to set the upper classification boundaries for GHS Class 4 test articles. The GHS limit concentrations may also be used for other inhalation toxicity studies. GHS discourages testing in excess of a limit concentration for animal welfare reasons. The limit concentration should only be exceeded when there is a compelling reason, and the reason should be explained in the study report. In the case of potentially explosive test articles, care should be taken to avoid conditions favourable for an explosion. It is generally advisable to not exceed 50% of the published Lower Explosive Limit (LEL) for safety reasons.

51. Achieving the GHS limit concentration of 5 mg/L is technically challenging for most aerosols and greatly exceeds real-world human exposure. It can be difficult or impossible to generate a respirable (MMAD of 1-4 µm) liquid or solid aerosol at this concentration without encountering experimental shortcomings. As aerosol concentration increases, particle size also increases due to the aggregation of solid particles or coalescing of liquid particles. The usual consequences are 1) a decrease in the respirable particle size fraction (and thus reduced toxicity), 2) increased fluctuation and variability in inhalation chamber concentrations accompanied by increased spatial inhomogeneities, 3) overloading of equipment used to characterize test atmospheres, and 4) a divergence of nominal and actual concentrations. At very high concentrations, dry powder aerosols and chemically reactive liquid aerosols (*e.g.*, polymers) tend to form conglomerates in the proximal nose causing physical obstruction of the animals' airways (*e.g.*, dust loading) and impaired respiration which may be misdiagnosed as a toxic effect. When testing aerosols, the primary goal should be to achieve a respirable particle size (MMAD of 1-4 µm). This is possible with most test articles at a concentration of 2 mg/L. Aerosol testing at greater than 2 mg/L should only be attempted if a respirable particle size can be achieved. As stipulated in TG 403, dilutions of corrosive test articles may be tested at exposure concentrations sufficient to extend the concentration-response curve to levels that reach the objective of the test and thus serve regulatory and scientific needs. These concentrations should be selected on a case-by-case basis and justification for concentration selection should be provided.

52. If the targeted regulatory limit concentration cannot be achieved by the initial technical procedures, then at least one alternative generation method should be used, ideally using different physical principles but established methodologies. A reasonable attempt should be made to generate the test article, but extreme technical solutions are not recommended. An explanation and supportive data should be provided that explains why the regulatory limit concentration could not be achieved. Information about a test article's toxicity can be derived from data about similar test articles or similar mixtures or products, taking into consideration the identity and percentage of components known to be of toxicological significance. If TG 403 is to be used, and there is little or no toxicity information, or if the test article is expected to be

toxic, a sighting study and a main study should be performed.

53. Those using the GHS Classification System should note that it uses units of mg/L to classify vapours, but units of ppm to classify gases even though gases and vapours are both gaseous when humans and animals are exposed to them. The conversion between mg/L and ppm is based on the molecular weight of a test article (see equations in paragraph 13). For example, at 22°C, 20 mg/L of a gas is equivalent to 24,200 ppm if the gas has a molecular weight of 20 g/mol, or 2420 ppm if it has a molecular weight of 200 g/mol. Gases and volatile test articles with a vapour saturation concentration that can exceed 20 mg/L (at approximately 22°C) should be tested at the limit concentration of 20 mg/L. This limit should only be exceeded when there is a compelling reason, and the reason should be explained in the study report. For volatile liquids with a vapour saturation concentration in the range of 2-20 mg/L (at approximately 22°C), the maximum chamber concentration should be at least in the range of this vapour saturation concentration. Commonly, this is achieved by generating a liquid aerosol, which then equilibrates with the vapour phase. Under such circumstances each phase needs to be appropriately collected and analyzed by the procedures used.

### **Performance of the Traditional protocol and the C×T Protocol**

54. Selection of the number of animals and the number of concentrations tested in the Traditional LC<sub>50</sub> protocol and the C×T protocol should be informed by the study director's understanding of the test's needed performance. A Performance Assessment of these two protocols is available at the OECD public website. It used simulated and real data sets to describe the strengths and weaknesses of both protocols, and the effect on point estimates that result from using an assortment of animal numbers, concentrations, and durations. Anyone who selects one of these protocols for a particular regulatory need is urged to carefully consider this landmark assessment (21).

55. Normally, two animals per C×T interval (one per sex using both sexes, or two of the more susceptible sex) will be adequate. The Performance Assessment simulation analysis, which tested 4 concentrations and 5 durations per concentration, demonstrated that performing a C×T protocol with 1 animal/sex or 2 animals of the more susceptible sex will provide LC<sub>50</sub> estimates that are comparable with a Traditional LC<sub>50</sub> protocol in terms of bias and precision. With 1 animal per sex (or 2 of the more susceptible sex) the performance with respect to LC<sub>10</sub> or LC<sub>01</sub> estimates is greater than one would expect from the Traditional LC<sub>50</sub> protocol, and reasonably reliable LC<sub>10</sub> or LC<sub>01</sub> estimates would usually be obtained for all durations within the tested time range (21). Under some circumstances, the study director may elect to utilize two rats per sex per C×T interval. The same simulation analysis demonstrated that testing 1 animal per sex per C×T combination may not be sufficient in all cases, even when testing 4 concentrations and 5 durations per concentration. Using 2 animals per sex per C×T interval (or 4 animals of the susceptible sex) may reduce bias and variability, increase the estimation success rate, and improve confidence interval coverage. If one is interested in the additional estimates available from a C×T experiment (*e.g.*, the one-hour LC values) not estimable from a 403 experiment, the addition of 1 extra animal per sex per C×T combination will reward the experimenter with better estimates (21). However, in case of an insufficient fit of the data (when using 1 animal per sex or 2 animals of the more susceptible sex per C×T interval) a 5<sup>th</sup> exposure with 5 durations may also suffice.

### **Selection of an Inhalation Chamber**

56. A dynamic, validated inhalation system with suitable control of all inhalation chamber parameters is required for acute inhalation toxicity studies. Dynamic inhalation systems include nose-only chambers and whole-body chambers. The default mode of exposure is nose-only (which term includes head-only, nose-only, or snout-only) for the following reasons:

- a) Exposure and/or uptake by the oral route (via preening) and dermal route are minimized, especially when testing aerosols.
- b) Technician exposure from handling exposed animals is minimized.
- c) A minimum of test article is needed due to low chamber volume.
- d) High concentrations (*e.g.*, limit concentrations) are readily achieved.
- e) The instability of test articles (*e.g.*, reactivity with excreta or humidity) and test atmosphere in-homogeneity are of minimal concern.
- f) The time required to attain chamber equilibration ( $t_{95}$ ) and decay is negligible relative to the duration of exposure and therefore not an issue.
- g) Adding or removing animal restraining tubes during exposure to a fixed steady state chamber concentration allows for multiple exposure durations in one single test (the C×T protocol, utilizing the same exposure concentrations for multiple exposure durations).
- h) The exposure of individual animals can be interrupted at any time during the course of exposure to avoid undue suffering of animals.
- i) Animals are readily accessible for specific physiological measurements (*e.g.*, respiratory function, body temperature) or the collection of blood, if applicable.
- j) The pre-conditioning of air prior to entering the inhalation chamber (*e.g.*, in order to eliminate ubiquitous environmental constituents such as ozone, nitrogen oxides, hydrocarbons, and particulates, or to allow testing under defined humidity or gas conditions) is technically less demanding with nose-only chambers than with larger whole-body inhalation chambers.

While nose-only is the default mode of exposure, special objectives of the study may be better achieved by using the whole-body mode of exposure. The use of non-default modes of exposure should be based on the focus of the study and should be justified in the study report.

57. In directed-flow (flow-past) nose-only inhalation chambers, the inhalation exposure air flow and the exhalation flow are separated so the exhaled air from one rat cannot be inhaled by another. Directed-flow chambers are preferable to chambers of small volume using a mixed-flow operation principle (24)(25) in which the inhalation exposure air flow and the exhalation flow can mix and be re-breathed. When an animal is confined to a restraining tube the observation of its behavior and physical condition is somewhat restricted. Subtle clinical signs may be obscured due to impaired locomotion and limited capability to evoke specific neurobehavioral responses. If the focus of a study is on neurobehavioral changes over the course of an exposure, this is sufficient justification for using an alternative exposure mode such as whole-body exposure. A detailed analysis and recording of clinical signs should be made, but not limited to, the time when maximal systemic toxicity is expected, which is usually on the exposure day. Advantages and disadvantages of each mode of exposure have been published (20). Because of the study design of the C×T protocol, a nose-only chamber should always be used.

### **Nose-Only Exposure Technique**

58. During exposure, animals are exposed to the test article while in restraining tubes. The restraining tubes should not impose undue physical, thermal, or immobilization stress on the animals. Restraint may affect physiological endpoints such as body temperature (hyperthermia) and/or respiratory minute volume. If generic data are available to show that no such changes occur to any appreciable extent, then pre-adaptation to the restraining tubes is not necessary. When precise dosimetry is the objective of the study, however, pre-adaptation may decrease inter-animal variability. Urine and faeces should escape from the restrainer during the course of exposure.



59. To provide optimal exposure of animals, a slight positive balance of air volumes supplied to and extracted from the exposure system should be ensured to prevent dilution of the test article at the animals' breathing zone. The design of the restraining tube and the pressure difference should make it impossible for animals to avoid inhalation exposure. If leakages from the inhalation chamber cannot be excluded by design, the inhalation chamber should be operated in a well-ventilated chemical hood to avoid harming laboratory personnel. Maintenance of slight negative pressure inside the hood will prevent leakage of the test article into the surrounding area.

60. Animals exposed in flow-past inhalation equipment designed to sustain a dynamic airflow that ensures an adequate air exchange of at least 2-3 times the respiratory minute volume of animals exposed (*i.e.*, at least 0.5 L/min per exposure port for rats). Each exposure port should have similar exposure conditions with an oxygen concentration of at least 19% and a carbon dioxide concentration not exceeding 1%. The design and operating conditions of the chamber should minimize the re-breathing of exhaled atmosphere. A significant disturbance of airflow dynamics during the collection of test atmosphere should be avoided.

### **Whole-Body Exposure Technique**

61. Animals should be tested with inhalation equipment designed to sustain a dynamic airflow of at least 10 air changes per hour. Higher airflow rates may be useful to meet specific requirements imposed by the test article. An oxygen concentration of at least 19%, a carbon dioxide concentration not exceeding 1%, and an evenly distributed exposure atmosphere should be ensured. Where concerns might apply, these gas levels should be measured in the vicinity of the animals' breathing zone. All animals should be individually housed to preclude them from breathing through the fur of their cage mates, thus reducing their aerosol exposure. To ensure stability of a chamber atmosphere, the total "volume" of the test animals should not exceed 5% of the chamber volume. Maintenance of slight negative pressure inside the chamber will prevent leakage of test article into the surrounding area. Food and drinking water should be accessible for exposures exceeding 8 hours.

62. In a dynamic whole-body chamber, the test article concentration initially rises rapidly, and then slowly approaches a theoretical equilibrium provided 1.) the output of the test article is constant and 2.) the test article is instantaneous and thoroughly mixed throughout the chamber. Under these conditions, an exponential built-up of concentration is seen throughout the chamber. The time to 95% atmosphere equilibrium ( $t_{95}$ ) in minutes is calculated using the following simplified formula:

$$t_{95} \text{ (min)} = 3 \times \left( \frac{\text{chamber volume}}{\text{chamber airflow}} \right)$$

More details are presented elsewhere (15).

## **MONITORING OF EXPOSURE CONDITIONS**

### **Chamber Airflows**

63. Airflow into dynamic inhalation chambers (e.g., pressurized air to disperse a test article, atmospheric air to evaporate a volatile test article, and dilution and conditioning airflows) and airflow at the chamber exhaust port should be controlled and monitored to obtain stable conditions throughout the exposure period. Pressure may also be measured within the chamber. Devices should be calibrated under conditions of use, e.g. by using bubble meters, wet test meters, dry gas meters, etc.. A technical description

of the calibration of devices that measure airflows should be documented and described in the study report. Further guidance is provided in paragraph 70.

#### **Chamber Temperature and Relative Humidity**

64. The chamber temperature should be maintained at  $22 \pm 3^{\circ}\text{C}$ . The relative humidity in the animals' breathing zone, for both nose-only and whole-body exposures, should be monitored regularly and recorded at least three times during each exposure. The relative humidity should ideally be maintained in the range of 30 to 70%, but this may not be possible when testing water based test articles, or may not be measurable due to test article interference with the test method. The proper performance of devices should be demonstrated, e.g. by using calibrated reference probes or saturated salt solution probes for measuring relative humidity. A technical description of the calibration of equipment used to measure inhalation chamber temperature and relative humidity, including the location of probes relative to the exposed animals, should be documented and described in the study report.

#### **Inhalation Chamber Sampling**

65. When assessing exposure concentrations (mass/volume of air), both the mass determined and the volumes of air sampled from the inhalation chamber and passed through the collection device should be identical and precisely measured. Flow meters, critical orifices, or dry gas meters used to define the sampled volume as a function of airflow ( $\text{rate} \times \text{time}$ ) should be appropriately calibrated. Sampled volumes can also be directly obtained with wet gas meters. Possible sampling errors, such as those caused by inappropriate collection efficiency, instability of the test article in solvents or on adsorbents, or a poor recovery from the collection medium, should be considered when designing a specific strategy to analyze components from inhalation chambers. Solvents evaporating from a collection device may cause volume errors. The collection efficiency depends markedly on the physical characteristics of the test article (gas, vapour, aerosol, particle size). Therefore, precautions should be taken to minimize size-selective sampling errors, and to assure that actual concentrations include all physical forms of the analyte examined.

66. Chamber atmosphere samples should be taken from the vicinity of the animals' breathing zone. During sampling, the airflow should be monitored at regular intervals to detect changes caused by an increased resistance in the adsorbent used. If impingers or gas bubblers containing volatile liquids (other than water) are used during sampling of test atmosphere, evaporation of the solvent should be taken into account. Sampling ports should be designed in such a way that potential sampling errors as a result of anisokinetic sampling or by size-selective sampling are minimized. The tolerance limits for the radius of the sample probes may be calculated according to published formulas (26)(27) or the relationship shown in Appendix I (Representative sampling of atmospheres). The collection efficiency of the equipment used to characterize exposure atmospheres should be measured. This information is of relevance when different devices used in a study provide inconsistent measurement results.

### **TEST ATMOSPHERE CHARACTERIZATION**

#### **Nominal Concentration**

67. Nominal concentrations (mass of test article disseminated into the exposure system during the generation period divided by the total airflow through the inhalation chamber during the same time period) and actual concentrations (measured mass concentration of test article recovered from the breathing zone of the exposed animal) should be determined. The nominal concentration is not used to characterize the animals' exposure. However, especially for gases or highly volatile substances, nominal concentrations are useful to judge the consistency of actual concentrations.

68. The consistency of inhalation tests can be judged by a comparison of nominal and actual concentrations for volatile liquid and gaseous test articles. However, this comparison is of limited

relevance for aerosols (solid or liquid) due to significant losses of particles in pre-separator systems and deposition on chamber and tubing walls. This is due to the fact that technically demanding measures should be taken for liquid and solid aerosols to remove large particle-size fractions from the air stream. Consequently, actual concentrations can significantly deviate from nominal concentrations, even by orders of magnitude. Ratios of nominal to actual concentrations are difficult to predict as they are contingent upon the apparatus used for aerosolization and particle size optimization, and they are dependent on the physico-chemical properties of the test article (e.g., viscosity, volatility, and ability to sublime or to co-distill with any carrier material). For liquid aerosols, the particle size distribution may decrease with the decreasing concentration. To achieve comparable particle size distribution within a wide range of concentrations (e.g., from 2 mg/L to 0.02 mg/L) dilution systems may be used. In this case the nominal concentration does not reflect the generation efficiency and is not meaningful.

### **Actual Concentration**

69. The actual concentration is the test article concentration at the animals' breathing zone in an inhalation chamber. Actual concentrations can be obtained either by specific methods (e.g., direct sampling, adsorptive or chemical reactive methods, and subsequent analytical characterisation) or by non-specific methods such as gravimetric filter analysis. The use of gravimetric analysis is acceptable only for single component powder aerosols or aerosols of low volatility liquids and should be supported by appropriate pre-study characterisation. Potentially reactive test articles should be assessed by methods specific for the test article that will not interfere with any degradation product. For single component test atmospheres, specific methods should be used for gaseous and volatile test articles, including liquid aerosols. Volatile test articles may exist as a vapour at low concentrations and as a vapour-aerosol equilibrium at higher concentrations. The applied sampling technology should integrate all phases. Non-specific methodologies may be appropriate for solid and liquid aerosols with low volatility provided the percentage of the vapour phase under testing conditions does not exceed 1% of the total concentration.

70. The exposure atmosphere shall be held as constant as practicable and monitored continuously and/or intermittently depending on the method of analysis. When intermittent sampling is used, chamber atmosphere samples should be taken at least twice in a four hour study. If marked sample-to-sample fluctuations occur, the next concentrations tested should use four samples/exposure. For very short exposure durations, the time required for atmosphere collection may exceed the animals' exposure duration. When testing very low aerosol concentrations, it may be technically difficult to accomplish this sampling frequency due to long sampling periods and the limited airflow rate typically used to extract samples from small inhalation chambers. Individual chamber concentration samples should deviate from the mean chamber concentration by no more than  $\pm 10\%$  for gases and vapours, and by no more than  $\pm 20\%$  for liquid or solid aerosols. In addition to the variability of chamber equilibrium concentrations, these error boundaries also comprise errors from other sources, e.g., variability related to the analytical method and variability in the sampling and collection of the analyte.

71. Ideally, analytical data obtained by intermittent sampling should be complemented by non-specific, real-time monitoring data (e.g., recorded by aerosol photometers for particulates or a total hydrocarbon analysers for volatile materials). These data can demonstrate that temporally stable exposure conditions prevailed, and that the time required to reach the inhalation chamber equilibrium concentration is negligible in relation to the total duration of exposure, or is adequately taken into account. Time to attain chamber equilibration ( $t_{95}$ ) should be calculated and reported. The duration of an exposure spans the time that the test article is generated. This takes into account the times required to attain chamber equilibration ( $t_{95}$ ) and decay. It should be noted that monitoring of the test atmosphere is an integral measurement of all dynamic inhalation chamber parameters and hence provides an indirect, though integrative, measure of inhalation chamber control. Therefore, the frequency of airflow measurements may be reduced to one single measurement at the start of an exposure. The characterization of test atmosphere should be representative for the atmosphere to which animals are exposed. Real-time monitoring instruments may not be suitable if their sensing units become covered with excessive quantities of test article or if they are

subject to being destroyed by the test article. If they cannot be used, expert judgement should be made as to whether the monitoring of physical chamber parameters generates relevant data. Care should be taken to avoid generating explosive concentrations.

72. For very complex mixtures consisting of vapours/gases, and aerosols (*e.g.*, combustion atmospheres and test articles propelled from purpose-driven end-use products/devices), both phases may behave differently in an inhalation chamber. Therefore, at least one indicator substance (analyte) normally the principal active in the tested product formulation, of each phase (vapour/gas and aerosol) should be selected. The back-calculation to the test article should utilize that analyte with the greatest precision, typically the one present in the highest concentration. For simple mixtures of known characteristics, *e.g.*, pesticide formulations, the gravimetric filter analysis should be given preference since this requires the least number of assumptions. It is not necessary to analyse inert ingredients provided the mixture at the animals' breathing zone is analogous to the formulation prior to aerosolization; the grounds for this conclusion should be provided by expert judgement. If there is some difficulty in measuring actual chamber concentration due to precipitation, non-homogenous mixtures, volatile components, or other factors, additional analyses of inert components may be necessary as detailed above.

73. Whenever the test article is a mixture (*e.g.*, a formulation), the analytical concentration should be reported for the total formulation and not just for the active ingredient or the component (analyte). In the case of simple mixtures, the percentage of potentially volatile components (*i.e.*, those presumed to be present as vapours in the inhalation chamber upon aerosolization of a liquid) relative to those components recovered by the filter should be determined. The mass concentrations obtained by filter analysis can then be back-calculated to the mass concentration of the test article. If gravimetric analysis is not suitable due to unstable gravimetric conditions (*e.g.*, continuous change in filter weight over a specified time of filter conditioning), the analysis of an appropriate component (analyte) of that mixture can then serve to back-calculate the actual test article concentration. If, for example, a simple mixture (*e.g.*, a pesticide formulation) contains 10% active ingredient and 90% inerts, the actual mixture concentration is the concentration of the active ingredient multiplied by ten. It is not necessary to analyze inert ingredients provided the mixture at the animals' breathing zone is analogous to that of the formulation. The grounds for this conclusion should be described in the study report.

### **Particle-Size Distribution**

74. Because aerosol particle size determines the deposition site in the respiratory tract, the particle-size distribution should allow for exposure of all relevant regions of the respiratory tract. Deposition and/or damage to any region of the respiratory tract may induce lethality, so it is not possible to predict, *a priori*, the most responsive region of the respiratory tract or the most harmful particle-size. Thus, aerosols with mass median aerodynamic diameters (MMAD) ranging from 1 to 4  $\mu\text{m}$  with a geometric standard deviation (GSD) in the range of 1.5 to 3.0 are recommended. Although a reasonable effort should be made to meet this standard, expert judgment should be provided if it cannot be achieved. For example, metal fumes may be smaller than this standard, and charged particles, fibers, and hygroscopic materials (which increase in size in the moist environment of the respiratory tract) may exceed this standard. It can be difficult for aerosols to meet this standard at high concentrations (*i.e.*, 5 mg/L) due to the tendency for solid aerosols to agglomerate and for liquid aerosols to coalesce (28).

75. Particle size analyses should use a mass-based metric that allows for direct comparison with mass-based actual concentrations. Multistage cascade impactors should be given preference. They should be designed to collect and classify the entire range of particle sizes present in the inhalation chamber that exceed approximately 0.1  $\mu\text{m}$ . Other devices or physical principles may be used if equivalence to the cascade impactor can be shown (with regard to MMAD and GSD, including the mass concentration sensed) or when required by the nature of the test article (*e.g.*, combustion atmospheres, smoke). Particle sizing should also be performed in test atmospheres where condensation aerosols may be formed from

vapour atmospheres. For non-adhesive aerosols, such as dry powders, the individual impactor stages should be covered with an adhesive stage coating (*e.g.*, silicone spray) if particle bounce and re-entrainment are expected. For high-concentrations of liquid aerosols, the stages may be covered by an adsorptive filter to prevent run-off of liquid deposits.

76. As shown in Appendix IV, the MMAD of the aerosol collected in the cascade impactor can be calculated using a "Cumulative Percent Found-Less Than Stated Particle Size" table. The steps are as follows:

- a) Calculate the total mass of test article collected in the cascade impactor. Start with test article collected on the stage that captures the smallest particle-size fraction (this would be the back-up filter if one is used), then divide this test article mass by the total mass found above.
- b) Multiply this quotient by 100 to convert to percent. Enter this percent opposite the effective cut-off diameter of the stage above it in the impactor stack. Repeat these steps for each of the remaining stages in ascending order.
- c) For each stage, add the percentage of mass found to the percentage of mass of the stages below it.
- d) Plot the percentage of the cumulative mass less than the stated size versus particle size using a log probability scale, and draw a straight line that best fits the plotted points (see Appendix IV). Established statistical procedures should be used to achieve the best fit.
- e) Note the particle size at which the line crosses the 50% mark. This is the estimated Mass Median Aerodynamic Diameter (MMAD).
- f) For calculation of ***Geometric Standard Deviation (GSD)*** refer to the log probability graph used to calculate the Mass Median Aerodynamic Diameter. Provided that the line is a good fit to the data, the size distribution is log-normal and the calculation of GSD is appropriate. Note the particle size at which the line crosses the 84.1% mark and the 50% mark. Calculate the geometric standard deviation (GSD) as follows.

$$GSD = \frac{84.1\% \text{ mark}}{50\% \text{ mark}}$$

- g) Algorithms for the calculation of particle size characteristics have been published (27)(28)(29)(30). A representative analysis of particle size data is shown in Table 1 and Fig. 2 (Appendix IV).

77. The mass concentration obtained by particle size analysis should be within reasonable limits of the mass concentration obtained by filter analysis. Equivalence demonstrates that there were no sampling errors (especially an under-sampling of larger particles) or particle losses within the device used to analyze particle size distribution. Non-equivalence in the presence of a highly loaded stage collecting the largest particle size might be taken as indirect evidence for the existence of particles too large to be collected by the device used to analyze particle size distribution.

78. In repeated inhalation exposure studies primary and secondary standard methods for the determination of actual concentrations may be used if primary standard results are confirmed by the simpler, less elaborate secondary standard methods.

## **Animal exposure**

### **Animal Selection and Assignment**

79. Animals are randomly selected, marked for individual identification, and kept in their cages for at least 5 days prior to the start of the test to allow for acclimatization to laboratory conditions. Although several mammalian test species may be used, the preferred species is the rat. Usage of common laboratory strains is recommended. If another mammalian species is used, the tester should provide justification for its selection. At the beginning of a study, young adult rats (approximately 8–12 weeks old) should be used (further details are given in paragraph 26).

80. The time interval between treatment groups is determined by the onset, duration, and severity of toxic signs. Commencement of an exposure should be delayed until one is reasonably confident of the outcome of previously treated animals. The exposure of animals at the next lower or higher concentration should be based on previous experience and scientific judgment.

### **Housing**

81. Each animal should be assigned a unique identification number. A system is required to randomly assign animals to test groups and a control group (if applicable). The animals may be group-caged by sex, but the number of animals per cage should not interfere with clear observation of each animal and should minimize losses due to cannibalism and fighting. The nature of a test article or toxic effects (*e.g.*, morbidity, excitability) may indicate a need for individual caging to prevent cannibalism. Animals should be housed individually in whole-body inhalation chambers during exposure to aerosols to prevent ingestion of test article due to grooming of cage mates. For feeding, conventional and certified laboratory diets may be used with an unlimited supply of municipal drinking water.

### **Exposure Time**

82. The duration of exposure should be specified. For whole-body chambers, the exposure time is defined as the time between the  $t_{95}$  equilibration of the chamber concentration and the  $t_{95}$  chamber concentration decay. Chamber equilibration and decay are assumed to be nearly instantaneously in nose-only chambers. For longer exposure durations, whole-body chambers are recommended.

### **Observations of Animals**

83. Animals should be observed frequently during the exposure period. Following exposure, careful clinical observations should be made at least twice on the day of exposure, or more frequently when indicated by the animals' response to treatment, and at least once daily thereafter during the postexposure period. Additional observations are made if the animals continue to display signs of toxicity. Observations include changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behavior patterns. Attention should be directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep, and coma. The measurement of rectal temperatures may provide supportive evidence of reflex bradypnea or treatment-/confinement-related hypo-/hyperthermia. Signs suggestive of mild neurotoxicity may be more difficult to observe in nose-only restrainers than in whole-body chambers. Guidance on clinical signs can be found in Chan and Hayes (31) and objective measurements that are indicative of impending death and/or severe pain and/or distress are available in OECD Guidance Document No. 19 (22).

84. The duration of the observation period is not fixed, but should be determined by the nature and time of onset of clinical signs and length of the recovery period. The times at which signs of toxicity appear and disappear are important, especially if there is a tendency for signs of toxicity to be delayed. All

observations are systematically recorded with individual records being maintained for each animal. Unless there are compelling reasons to do otherwise, animals found in a moribund condition and animals showing severe pain and/or enduring signs of severe distress should be humanely killed without delay for animal welfare reasons. When animals are killed for humane reasons or found dead, the time of death should be recorded as precisely as possible.

85. Care should be taken when conducting examinations for clinical signs of toxicity that initial poor appearance and transient respiratory changes, resulting from the exposure procedure, are not mistaken for treatment-related effects. Animals killed in a moribund state are considered in the interpretation of the test results in the same way as animals that died on test. Some test articles may have effects with delayed onset, such as an obliterating bronchiolitis. Animal welfare aspects, and the likelihood of scientific misjudgement, need to be carefully balanced. Expert judgment is needed to justify the respective procedure.

### **Body Weight**

86. Individual animal weights should be recorded on the day of exposure prior to exposure (day 0), and at least on days 1, 3, and 7 (and weekly thereafter), and at the time of death or euthanasia if exceeding day 1. Surviving animals are weighed and humanely killed at the end of the post-exposure period. Animals should be observed for a minimum of 14 days. Extended observation periods may be necessary if toxic effects fail to reverse or are delayed in onset. A sustained decrement in body weight is recognized as a critical indicator of moribundity and should therefore be closely monitored. At the end of the test, surviving animals are weighed and then humanely killed.

### **Pathology**

87. All test animals, including those which die during the test or are removed from the study for animal welfare reasons, should be subjected to complete exsanguination (if feasible) and gross necropsy. Necropsies should be performed as soon as possible. If a necropsy cannot be performed immediately after a dead animal is discovered, the animal should be refrigerated (not frozen) at temperatures low enough to minimize autolysis. All gross pathological changes should be recorded for each animal with particular attention to any changes in the respiratory tract. Determination of lung weight and microscopic examination may be considered for organs showing evidence of gross pathology in animals surviving 24 or more hours. Microscopic examination may also be considered for the respiratory tract if it is likely to be affected because it may yield useful information, such as evidence of irritation. For test articles that may cause tissue destruction at the site of initial deposition within the respiratory tract, microscopic examination of the entire respiratory tract should be considered. Tissues should be adequately fixed and the examination should include sections of the nasal tissues, larynx, trachea, main bronchi, and lung lobes. Microscopic examination of these tissues may provide useful information on the test article's pattern of deposition within the entire respiratory tract and mode of action.

### **Respiratory Physiology and Bronchoalveolar Lavage**

88. As described above, specific regulatory requirements may require sublethal endpoints in addition to lethal endpoints. Measurements of breathing patterns (*e.g.*, respiratory rate and tidal volume) prior to, during, and/or shortly after exposure may provide useful information for estimating the relative irritant potency of inhaled agents and for determining whether an inhaled test article is an upper or lower respiratory tract irritant. Lung lavage may provide useful quantitative information for concentration-response changes. Serial bronchoalveolar lavage (BAL), which requires additional animals, may provide additional information on time-course changes following single exposures. Analysis of arterial blood gases may also be useful to assess perfusion:ventilation imbalance of the lung.

89. When there is evidence that the lower respiratory tract (*i.e.*, the alveoli) is the primary site of deposition and retention, then BAL may be the technique of choice to quantitatively analyse hypothesis-

based dose-effect parameters focusing on alveolitis, pulmonary inflammation, and phospholipidosis. This allows for dose-response and time-course changes of alveolar injury to be suitably probed. BAL measurements are particularly useful when the response is generalized and occurring in the luminal parts of the lower respiratory tract, but it may also be of limited relevance for focal responses or areas/interstitial responses not accessible by the lavage fluid. Because severe inflammation can lead to airway plugging, the most severely damaged location may not be accessible via the lavage fluid. Thus, BAL measurements generally complement the results from histopathology examinations but cannot replace them.

90. Although lung lavage can be performed in many ways, one suitable method is the following: After exsanguination, the lung is excised, weighed, and lavaged. If the focus of histopathology is on tissue-related changes then the lavaged lung is instilled by an appropriate fixative using an instillation pressure of 20-30 cm of water and further processing. Histopathology on lavaged lungs may be considered disadvantageous under certain circumstances. Expert judgement is needed to justify whether to use extra animals for histopathology or to use partial lavage procedures. When doing the latter, one half of the lung is tied off and then used for weighing and/or histopathology as described above (in case of rats commonly the left lung lobe), and the other half (lobus cranialis, l. medius, l. caudalis, l. accessorius) is lavaged. Thus, studies with a focus on characterizing a NOAEL should preferentially use the first procedure. Whenever the analysis is more qualitative (*e.g.*, mechanism-related or proof-of-principle-related), the second option may be preferable for animal welfare reasons.

91. The BAL fluid may be analysed for total and differential leukocyte counts, total protein, and lactate dehydrogenase. Other parameters that may be considered are those indicative of lysosomal injury, phospholipidosis, fibrosis, and irritant or allergic inflammation which may include the determination of pro-inflammatory cytokines/chemokines.

92. With regard to repeated exposure studies additional considerations are required:

- A. In case any vehicle is used (other than water) the vehicle may not interfere with expected toxicological effects. The range of reference values as defined by historical air controls may not be exceeded. In order to utilize data from vehicle studies to establish a historical data base, vehicle concentrations need to be characterized analytically. It is not permissible to utilize historical vehicle control data based on 'nominal concentrations' alone.
- B. Particle size may be smaller than 1 µm for some test articles, *e.g.*, smoke, nano-particulates or other substances technically designed to be smaller than 1 µm. Especially for particles in the size range  $\leq 0.1$  µm specialized equipment for particle size analysis is required. For submicron sized particle size analysis the count median diameter rather than the mass median diameter needs to be reported. In case non-isometric structures are tested, modified procedures revealing the geometric length, diameter and aspect ratios, in addition to the aerodynamic properties, should be considered.
- C. Day-to-day fluctuations in concentrations and particle size need to be considered in repeated exposure studies. Real-time monitoring devices should be used to complement time-weighted average samples to demonstrate that temporal stability of concentrations has been attained and that short-term peak excursions do not occur.
- D. More comprehensive analytical verification of complex atmospheres consisting of multiple components should be considered and whether inhalation chamber atmospheres are homogeneous at different inhalation chamber locations/exposure ports.
- E. Due to the testing of very low concentrations and the higher number of experimental animals per chamber (relative to acute studies) possible interferences / interactions of the test article with



excreta/ammonia/exhaled air etc. needs to be considered.

- F. Opposite to non-inhalation studies animals are physically handled twice a day, before and after exposure. Unique marking systems (*e.g.*, subcutaneously implanted transponders) should be used to exclude a mix-up of animals.

## VII. STATISTICAL ANALYSIS OF DATA

### Median Lethal Concentration (LC<sub>50</sub>) and Fractional Percentages

93. Dosage-effect relationships can usually be described by cumulative frequency distributions, mathematically represented by sigmoid curves. For each substance, a dosage (concentration)-effect relationship exists which is assumed to be characteristic for a specific effect and species. In order to quantify this relationship, the term "median lethal concentration" (LC<sub>50</sub>) was suggested as a measure of acute inhalation toxicity. The median lethal concentration is defined as the concentration that kills half of a suitably large number of animals exposed for a specified duration. Determination of the LC<sub>50</sub> requires a mathematical description of the concentration-effect curve. Hence, the concentration-effect curve can be suitably transformed into a linear function by a log-concentration probit-cumulative mortality relationship. Other mathematical transformations that have been employed to linearize the concentration-effect curve include the use of the logistic function, angular transformation, and moving averages and interpolation (32)(33)(34).

94. The prerequisite to calculating the median lethal concentration or fractional percentages thereof is the availability of the following data:

- Actual exposure concentrations
- The number of animals exposed
- The number that died.

In tests with few animals per exposure level the Thompson's method of moving averages may be the most efficient methodology and will give a sufficiently accurate solution if equally spaced test concentrations are used. If, however, one wishes to estimate a number of fractional toxicity levels (LC<sub>01</sub>, LC<sub>10</sub>, ...) and is interested in more precisely establishing the slope of the concentration/lethality curve, sufficient exposures levels with the log/probit regression technique are required. The method used should allow the calculation of 95% confidence intervals at any point on the regression line. Tests of significance between two or more such sets of data (*i.e.*, slopes of mortality curves) may readily be done by t-type tests. Note that the confidence interval at any one point will be different from the interval at other points and should be calculated separately. Additionally, the nature of the probit transform is such that toward the extremes-LC<sub>01</sub> and LC<sub>99</sub>, for example, the confidence intervals will "balloon," that is, they become very wide. Because the slope of the fitted line in these assays has a very large uncertainty in relation to the uncertainty of the LC<sub>50</sub> (the midpoint of the distribution), a great deal of caution should be exercised with calculated LC<sub>x</sub> values.

95. When experimental/mathematical procedures require the estimation of median lethal concentration values from multiple exposure durations (LC<sub>t50</sub>) this is accomplished by the C×T protocol combining the exposure concentration (C), exposure time (t) and the toxic load exponent (n), using the following equation:  $k = C^n \times t$  where k is a numerical constant (34)(35). This equation can be generalized using a two variate surface plot relating toxicity (mortality) and time as follows:

$$y = b_0 + b_1 \log(C) + b_2 \log(t)$$

$$\text{where } n = b_1/b_2$$

Here, y is either the Probit or the Normit value and b<sub>0</sub>, b<sub>1</sub> and b<sub>2</sub> are empirically derived constants. It should be recognized that C does not have inherent exponential properties, but t might have such properties because toxicity, under non-ideal conditions, is a function of at least two independent time-scales, one being the half-life of the rate-determining step of the intoxication, and the other being the intensity of exposure. When sufficient data are available, the empirical constants shown above can be suitably solved mathematically by iterative mathematical procedures combining all C×T relationships

evaluated in one single matrix. From the constants of the two variate surface plot, the respective  $LCt_{50}$  and  $LCt_{01}$  (or any other values), including their confidence intervals, can readily be estimated. Short exposure times (less than 15 minutes) may lead to a transiently decreased inhaled dose after onset of exposure and, accordingly, underestimation of toxicity. Therefore, trigger values estimated from  $C \times T$  relationships preferentially based on exposure durations of less than 15 minutes should be judged cautiously.

### **Body Weights and Non-Lethal Endpoints**

96. Among the sets of data commonly collected in acute inhalation studies are body weights, the weights of selected organs, body temperature, and selected clinical pathology parameters in studies where the focus is on non-lethal endpoints. In fact, body weight (or the rate of body weight gain) is frequently the most sensitive indication of an adverse effect. How to best analyze this, and in what form to analyze the organ weight data (as absolute weights, weight changes, or percentages of body weight), have been dealt with elsewhere (32). Both absolute body weights and body weight gains (calculated as changes from a baseline measurement value which is traditionally the animal's weight immediately prior to the exposure to test material) are almost universally best analyzed by ANOVA followed, if called for, by a *post hoc* test. Comparisons should be made against equally exposed historical control groups. Due to sequential exposure sessions, shifts in baseline body weights across exposure groups are inevitable in acute inhalation studies. Therefore, the statistical analysis of body weight gains should be given preference. The advantage is an increase in sensitivity because the adjustment of starting points (the setting of initial weights as a "zero" value) acts to reduce the amount of initial variability. In this case, Bartlett's test is performed first to ensure homogeneity of variance and the appropriate sequence of analysis follows. With smaller sample sizes, the normality of the data becomes increasingly uncertain, and nonparametric methods such as Kruskal-Wallis may be more appropriate (32)(36).

97. The analysis of clinical pathology data is best analyzed by ANOVA followed, if called for, by a *post hoc* test. Repetitively measured data should preferentially be analyzed by a one-way repeated measures analysis of variance (RM-ANOVA). All data are then compared against the pre-exposure data, if applicable. For data that pass the normality and equal variance tests, the multiple comparisons Dunnett's *post hoc* procedure is used to isolate the time points that differ from pre-exposure data. The criterion for statistical significance should be  $P < 0.05$ . Some concentration-effect relationships may be associated with concentration-dependent increase in variability. This can reasonably be compensated for by the logarithmic transformation of data. Endpoints which are 'zero' prior to exposure or in the control should be transformed prior to analysis using the arcsine square-root function. This transformation is appropriate for percentages and proportions because the transformed data more closely approximate a normal distribution than do the non-transformed proportions (37).

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## APPENDIX I

### GLOSSARY OF TERMS

**Absolute temperature:** The absolute temperature (T) at 0 °C is 273.15 Kelvin [°K]. Thus, T [°K] = 273.15 + degrees Celsius.

**Actual concentration:** The concentration of a test article in the test animal's breathing zone. The sampled mass of the test article is determined by characterizing one or more constituents using either an analytical method specific for a selected component (e.g., chromatography) or a nonspecific, integrating method which addresses all non-volatile components, such as the total mass obtained by filter analysis (see gravimetric concentration). The terms actual concentration and analytical concentrations are commonly used interchangeably. The analytical or gravimetric concentration (not the nominal concentration) is generally used for hazard assessment. The actual concentration is commonly expressed in mass units per unit volume of air (mg/L, mg/m<sup>3</sup>). The mass of test article per unit mass of test animal (e.g., mg/kg), or inhaled dose, is difficult to define in inhalation toxicity studies since the fraction of test article deposited/absorbed/retained in the respiratory tract is dependent on a number of variables often not defined or measured in acute inhalation studies. Due to these uncertainties, exposure should be defined in terms of the "actual exposure concentration" and not the "exposure dose".

**Acute inhalation toxicity:** The adverse effects caused by an airborne test article following a single uninterrupted inhalation exposure of less than 24 hours. Most acute inhalation toxicity studies are 4 hours in duration.

**Aerodynamic diameter:** The diameter of a unit density sphere having the same terminal settling velocity as the particle in question, whatever its size, shape, and density. It is used to predict where in the respiratory tract such particles may be deposited (24).

**Aerodynamic particle sizer:** A particle spectrometer that uses an acceleration system to differentiate particles by aerodynamic diameter and a laser velocimeter to detect particles (24). See also **Cascade impactor**.

**Aerosol:** A relatively time-stable suspension of small solid or liquid particles in a gas. The diameter size range of aerosol particles is about 0.001 to 100 µm (24). See also **dust, fog, fume, haze, mist, smog, and smoke**.

**Agglomerate:** A group of particles held together by van der Waals forces or surface tension (24).

**Aggregate:** A heterogeneous particle in which the various components are not easily broken apart (24).

**Alveolar:** The portion of the respiratory system in which gas exchange occurs; alveoli are small sacs at the end of the bronchioles.

**Analytical concentration:** See actual concentration.

**Aspiration efficiency:** The fraction of particles entering an inlet from an inhalation chamber. Anisokinetic sampling losses may cause the aspiration efficiency to be less than 1.

**Atomizer:** A device used to produce liquid aerosols by mechanical disruption of a bulk liquid. Usually this consists of a metering pump connected to a nozzle.

**Attrition:** The wearing down of coarse powders and granules into airborne dust due to mechanical abrasion or stress.

**Bubble meter:** A tube with a defined volume into which bubbles are injected to measure airflow rate.

**Cascade impactor:** A device that uses a series of impaction stages with decreasing particle cut size so that particles can be separated into relatively narrow intervals of aerodynamic diameter; used to measure

aerodynamic particle size (24).

**Coagulation:** An aerosol growth process resulting from the collision of aerosol particles.

**Concentration:** The mass of test article per unit volume of air (*e.g.*, mg/L, mg/m<sup>3</sup>), or the unit volume of test article per unit volume of air (*e.g.*, ppm, ppb).

**Corrosivity:** Test article-induced destruction of tissue at the portal-of-entry (*e.g.*, oral, dermal, ocular, inhalation). Test articles defined as corrosive to gastrointestinal, dermal, or ocular tissues may not necessarily be corrosive to the respiratory tract. Because corrosivity in the respiratory tract may be site specific, the identification of affected sites may provide important information.

**Critical orifice:** An orifice through which there is a constant flow when a sufficient pressure drop across the orifice causes sonic flow (27).

**Cyclone:** A mechanic device shaped as a conical cylinder that uses geometry and centrifugal acceleration to separate suspended particles from a gaseous stream on the basis of aerodynamic particle size.

**Dust:** Dry solid particles dispersed in a gas as a consequence of mechanical disruption of a bulk solid material or powder formed from a single component or mixture. Dust particles are generally irregular and larger than 0.5 µm (27).

**Dynamic inhalation chamber:** A type of push-and-pull inhalation chamber with a constant airflow in which the atmosphere and test article are held constant so that inhalation chamber equilibrium is attained. Unlike a static chamber which has no airflow, a dynamic chamber has a steady state test article concentration, oxygen concentration, carbon dioxide concentration, temperature, and relative humidity for the duration of the exposure period. See also **Equilibrium concentration**.

**Effective Cut-off Diameter (ECD):** The upper particle size limit for a given stage of a cascade impactor.

**Elutriator:** A device used to separate fine particles from large particles.

**Equilibrium concentration:** In dynamic systems, the test atmosphere is continuously delivered to and exhausted from the animal exposure chamber in a flow-through manner; the test article is not recirculated. After an initial rise, the chamber concentration will approach and maintain a stable equilibrium concentration if the air flow rates (in/out) and the generation rate are constant. Prediction of this equilibrium concentration requires accurate information on generation rate, losses of test article in various parts of the system, and flow rates as exemplified by the following formula:

$$C_t = C_0 \left( 1 - e^{-\frac{F}{V}t} \right)$$

where  $C_t$  = concentration at the time  $t$ ,  $C_0$  = equilibrium chamber concentration,  $F$  = total flow through the chamber, and  $V$  = chamber volume. For practical purposes, the inhalation chamber equilibrium is attained at the time  $t_{95}$  which is when  $C_t = 95\% C_0$ .

**Equivalence diameter:** The median equivalence diameter may reflect the number of particles, as in the count median diameter (CMD), reflect the mass, as in the mass median diameter (MMD), or reflect the volume, as in the volume median diameter (VMD). Small particles (< 0.5 µm) diffuse like gases and are defined by diffusion-equivalence diameter (thermodynamic), while larger particles respond to inertial forces and are defined by aerodynamic diameter.

**Evaporation:** 1. The transition from the liquid phase to the vapour phase. 2. The condition in which more vapour molecules are leaving a particle's surface than arriving at the surface, resulting in shrinkage of a liquid particle. See also **Sublimation**.

**Exposure chamber:** A closed system used to expose animals to a gas, vapour, or aerosol of a test article.



See **Dynamic inhalation chamber**, **Nose-only inhalation chamber**, and **Whole-body inhalation chamber**.

**Extrathoracic:** The portion of the respiratory tract before the thorax including the nose, mouth, nasopharynx, oropharynx, laryngopharynx, and larynx.

**Finney equation:** This established relationship may be used to estimate an  $LC_{50}$  for a mixture, provided all components produce additive acute toxicity and have parallel regression lines of probit against log doses (19).

$$\frac{1}{LC_{50}} = \left( \frac{\% \text{ ingredient}_1}{LC_{50_1}} \right) + \left( \frac{\% \text{ ingredient}_2}{LC_{50_2}} \right) + \dots + \left( \frac{\% \text{ ingredient}_n}{LC_{50_n}} \right)$$

**Fog:** A dense mist which impairs visibility. It is typically formed by condensation of supersaturated vapour. See also **Mist**.

**Friable:** Solid material easily crumbled. See also **Attrition**.

**Fume:** Small solid particles that are usually the result of condensed vapour, with subsequent agglomeration. Fumes are often the result of combustion, welding, and other high temperature processes (27).

**Gas:** The state of matter distinguished from the solid and liquid states by relatively low density and viscosity, relatively great expansion and contraction with changes in pressure and temperature, the ability to diffuse readily, and the spontaneous tendency to become distributed uniformly throughout any container.

**Geometric standard deviation ( $\sigma_g$  or GSD):** A unitless number used to portray the range of particle sizes. A particle distribution is considered to be monodisperse when the  $\sigma_g$  is 1.0-1.2, and polydisperse when the  $\sigma_g$  is  $>1.2$  (38).

**GHS Globally Harmonized System of Classification and Labelling of Chemicals:** A system for the classification of chemicals according to standardized types and levels of physical, health and environmental hazards, and addressing corresponding communication elements, such as pictograms, signal words, hazard statements, precautionary statements and safety data sheets, so as to convey information on their adverse effects with the intent to protect people and the environment. A joint activity of OECD (human health and the environment), UN Committee of Experts on Transport of Dangerous Goods (physico-chemical properties) and ILO (hazard communication) and co-ordinated by the Interorganisation Programme for the Sound Management of Chemicals (IOMC).

**Gravimetric concentration:** An inexpensive integrating method for measuring total aerosol concentrations in which test atmosphere sampled from the animals' breathing zone is passed through a filter system. The total gravimetric concentration is calculated by dividing the mass of test article collected on the filter by the volume of air passed through the filter. Although gravimetric measurements are acceptable for dusts and liquids with low vapour pressures, other sampling and analytical methods (such as GC, HPLC, etc) should be used to measure chamber concentrations of gases, vapours, and liquids with moderate to high vapour pressures. Especially for moderately volatile test articles which exist as an equilibrated atmosphere of a liquid aerosol or dust (sublimation) and a vapour phase, the collection principle and the analytical determination should integrate all phases of a specific component.

**Haber's rule:** The relationship between concentration and time to response for any given chemical is a function of the physical and chemical properties of the test article and the unique toxicologic and pharmacologic properties of the individual test article. The relationship according to Haber is  $C \times T = k$ , where  $C$  = actual exposure concentration,  $t$  = exposure duration ( $\geq t_{95}$ ), and  $k$  = a constant). This concept states that exposure concentration and exposure duration may be reciprocally adjusted to maintain a cumulative exposure constant ( $k$ ) and that this cumulative exposure constant will always reflect a specific

quantitative and qualitative response. This relationship can also be expressed by the equation  $C^n \times t = k$ , where  $n$  represents a chemical-specific, and even a toxic endpoint specific, exponent. The relationship described by this equation is basically in the form of a linear regression analysis of the log-log transformation of a plot of  $C$  vs.  $t$ . Ten Berge et al. (39) found that the empirically derived value of  $n$  ranged from 0.8 to 3.5 among a group of chemicals examined.

**Haze:** A combination of vapour, dust, fume, and mist.

**Humane end point:** A humane endpoint can be defined as the earliest indicator in an animal experiment of severe pain, severe distress, suffering, or impending death.

**Impending death:** When a moribund state or death is expected prior to the next planned time of observation. Signs indicative of this state in rodents could include convulsions, lateral position, recumbence, and tremor (see the Humane Endpoint Guidance Document (22) for more details).

**Impinger:** A device in which particles are removed by impacting aerosol particles into a liquid.

**Inhalable aerosol:** Fraction of an aerosol that can enter the human respiratory system through the nose and mouth.

**Inhalable diameter:** The aerodynamic diameter of particles which can be inhaled through the nose and/or mouth of a given organism and deposited anywhere along the respiratory tract.

**Inhalation:** Exposure to a test article by normal respiration. The entire respiratory tract can be exposed.

**Inhalation chamber equilibrium:** see **Equilibrium concentration**.

**Isokinetic sampling:** Sampling condition in which the air flowing into an inlet has the same velocity and direction as the air flow at the sample collection point (see also **Representative sampling of atmospheres**).

**Kelvin effect:** Increase in partial vapour pressure for a particle's curved surface required to maintain mass equilibrium relative to the vapour pressure above a flat liquid surface. This means that molecules tend to evaporate faster from small particles than from a flat liquid surface (see also vapour).

**Kelvin:** see **Absolute temperature**.

**LC<sub>50</sub> (median lethal concentration):** A time dependent, statistically derived estimate of a test article concentration that can be expected to cause death during exposure or within a fixed time after exposure in 50% of animals exposed for a specified time. The LC<sub>50</sub> value is expressed as mass of test article per unit volume of air (mg/L, mg/m<sup>3</sup>) or as a unit volume of test article per unit volume of air (ppm, ppb). The exposure duration should always be specified (*e.g.*, 4-hour LC<sub>50</sub>).

**LCt<sub>50</sub> (median lethal concentration per minute):** The product of the concentration of a toxic gas, vapour, or aerosol and the exposure time causing lethality in 50% of test animals. For details see **LC<sub>50</sub> (median lethal concentration)**. The LCt<sub>50</sub> is expressed as mg/m<sup>3</sup>•min.

**Limit concentration:** The maximum concentration required for an inhalation toxicity study, depending on the physical state of the test article. When the GHS Classification System is used, the limit concentrations for gases, vapours, and aerosols are 20,000 ppm, 20 mg/L and 5 mg/L, respectively, (or the maximum attainable concentration).

**Limit test:** An inhalation toxicity study performed using a single group of animals exposed to the test-specific limit concentration.

**Mass median aerodynamic diameter (MMAD):** Mass median of the distribution of mass with respect to aerodynamic diameter. The median aerodynamic diameter and the geometric standard deviation are used to describe the particle size distribution of an aerosol, based on the mass and size of the particles. Fifty percent of the particles by mass will be smaller than the median aerodynamic diameter, and 50% of the particles will be larger than the median aerodynamic diameter. MMADs of 1-4 µm are recommended for

acute inhalation toxicology studies. See also **Equivalence diameter**.

**Maximum attainable concentration:** For vapour atmospheres, this concentration depends on the vapour saturation concentration of a test article under test conditions. For liquid and solid aerosols this concentration depends on a test article's physical properties and also the type of equipment used to generate the aerosol. The maximum attainable concentration is generally defined such that any change of equipment and/or further increase of the nominal test article supply rate into the inhalation exposure system does not increase the concentration of respirable aerosol to any appreciable extent.

**Micronization:** Mechanical procedure to reduce particle size. Mechanical stress due to milling, grinding or breakdown of particles may produce artifacts, such as surface activation and test article degradation.

**Mist:** A liquid aerosol, typically formed by condensation of supersaturated vapours or by physical shearing of liquids, such as in nebulization, spraying, or bubbling (24). A dense mist which impairs visibility is called a **fog**.

**Mixtures:** see **Test article**.

**MMAD:** See **Mass Median Aerodynamic Diameter**.

**Monodisperse aerosol:** Particles that are uniform in size. For practical purposes, an aerosol with a GSD < 1.2 may be considered monodisperse (38). See also **Polydisperse aerosol** and **Geometric Standard Deviation**.

**Moribund status:** Being in a state of dying or inability to survive, even if treated. (See the Humane Endpoint Guidance Document (22) for more details).

**Nebulizer:** A device in which droplet aerosols are produced by dispersion of a bulk liquid in a system that allows larger particles to be impacted and smaller particles to escape from the system (*e.g.*, collision nebulizer).

**Nominal concentration:** The concentration of test article introduced into a chamber system. It is calculated by dividing the mass of test article generated by the volume of air passed through the chamber. The nominal concentration does not necessarily reflect the concentration to which an animal is exposed. The resultant **actual concentration** cannot be predicted from the nominal concentration by default because of its dependence on laboratory-specific technical variables. See also **Actual concentration**.

**Nose-Only Inhalation Chamber:** An inhalation chamber system that minimizes dermal exposure and oral exposure (via licking of contaminated fur). Animals are placed in a restraining tube during the course of exposure. The design of this tube should not interfere with the thermoregulation of the animal to any appreciable extent. **Head-only** and **snout-only** are synonyms of nose-only.

**Pascal:** A unit of pressure used to define atmospheric pressure and vapour pressure. It is interrelated to other pressure units as follows:  $1 \text{ Pa} = 10^{-5} \text{ bar} = 0.987 \cdot 10^{-5} \text{ atm} = 0.0075 \text{ Torr}$ .

**Particle bounce:** The rebounding of particles that fail to adhere after impacting on the collecting surface of a cascade impactor stage. Compare with **Re-entrainment**.

**Particle size** - see **Aerodynamic particle size**.

**Particle size distribution:** A description of how much of an aerosol is in each of a set (or continuum) of size intervals.

**Polydisperse aerosol:** An aerosol composed of particles with a range of sizes. A particle distribution is considered to be monodisperse when the GSD is 1.0-1.2, and polydisperse when the GSD is >1.2 (32). See also **Monodisperse aerosol** and **Geometric Standard Deviation**.

**Portal-of-entry effect:** A local effect produced at the tissue or organ of first contact between the toxicant and a biological system. For the inhalation route, the portal-of-entry can be any part of the respiratory tract

from the nose to the terminal alveoli of the lung.

**Pulmonary (PU):** Pertaining to the lungs, including the respiratory bronchioles, alveolar ducts, and alveoli.

**Preparation:** Formulation of multiple components. See **Test article**.

**Re-entrainment:** Return of particles to an air stream after deposition on a collecting surface of a cascade impactor stage. Compare with **Particle bounce**.

**Relaxation time:** Relaxation time is a parameter used to describe the settling behaviour of particles. The gravitational force effectively removes larger particles from the suspending gas.

**Representative sampling of atmospheres:** Tolerance limits for the sample probe orifice ( $r_p$ ) can be calculated using formulas with varying complexity (24) in order to obtain optimal inlet efficiency for a specified sampling flow rate. The inlet efficiency is the fraction of airborne particles that is delivered to the aerosol transport section of a sampling system by the inlet. It is the product of the aspiration and transmission efficiencies. The formula shown below may be applicable to most conditions utilized in inhalation toxicology (at 293.15 Kelvin, 101.3 kPa, particles suspended in relatively calm air). This formula is arbitrarily selected and other, more complex formulas also may be more applicable for specialized purposes.

$$5 \times 3 \sqrt[3]{\frac{\text{flow} \times \tau}{4 \times \pi}} \leq r_p \leq \frac{1}{5} \times 2 \sqrt[3]{\frac{\text{flow}}{g \times \tau \times \pi}}$$

$r_p$  = radius of the sample probe ( $r_p$ ) in cm; flow = flow rate ( $\text{cm}^3 \times \text{sec}^{-1}$ ),  
 $\tau$  = relaxation time (sec),  $g$  = gravity constant =  $980 \text{ cm/sec}^2$

#### **Example calculation:**

The targeted sampling airflow rate from an inhalation chamber is 3 L/min ( $50 \text{ cm}^3/\text{sec}$ ) and the probe sampling collection efficiency needs to be considered for particles up to  $20 \mu\text{m}$ . Under these conditions the relaxation time for the largest particle of interest is approximately 0.001 sec.

$$5 \times 3 \sqrt[3]{\frac{50 \times 0.001}{4 \times \pi}} \leq r_p \leq \frac{1}{5} \times 2 \sqrt[3]{\frac{50}{g \times 0.001 \times \pi}} \Rightarrow 0.79 \leq r_p \leq 0.81 \text{ cm}$$

On the other hand, for particle up  $15 \mu\text{m}$  (relaxation time  $6 \times 10^{-4}$ ) the inlet radius should meet the following conditions:  $0.67 \leq r_p \leq 1.04 \text{ cm}$ . These examples show that larger particles may not be sampled representatively if the sampling flow rate relative to the probe diameter does not match the required relationship.

**Respirable diameter:** The aerodynamic diameter of particles which are capable of reaching the gas-exchange region in the lungs (the alveoli) for the organism under study.

**Respirable fraction:** Fraction of aerosol that can reach the gas exchange region of the respiratory system (i.e., the alveoli). For details see European Standard EN 481:1993 (39).

**Respirable particulate mass:** The mass of material that is deposited in the gas-exchange region of the lungs for the organism under study.

**Retention:** The amount of deposited particles that are not cleared from the respiratory tract at a particular time after exposure.

**Rotameter:** An airflow rate meter.

**Sedimentation:** Movement of particles by the influence of gravity.

**Sighting study:** A preliminary study performed using a minimum of animals for the purpose of selecting concentrations to be used in a main study.

**Smog:** A word combination of smoke and fog; a combination of gases and aerosols formed during UV irradiation of hydrocarbons and oxides of nitrogen, ozone, etc.

**Smoke:** A solid and/or liquid aerosol which is the result of incomplete combustion or condensation of supersaturated vapour. Most smoke particles are sub-micrometer in size.

**Static inhalation chamber:** An inhalation chamber without a source of fresh air. Static chambers cannot be used for Guideline studies because test article and oxygen concentrations decrease, and carbon dioxide concentration, humidity, and chamber temperature increase as the study progresses. Compare with **Dynamic inhalation chamber**.

**Sublimation:** 1. The transition from the solid phase directly to the vapour phase without passing through a liquid phase (*e.g.*, dry ice). 2. The condition in which more vapour molecules are leaving a solid particle's surface than arriving at the surface, resulting in shrinkage of the particle. The opposite of sublimation is **Deposition**.

**Target concentration:** The desired chamber concentration. See also **Nominal concentration** and **Actual concentration**.

**Test substance:** see **Test article**.

**Test article:** A product, substance, preparation or mixture (a formulation of multiple components) used for inhalation testing. Some test articles may be thermally decomposed for the purpose of testing, as in combustion toxicology tests. Atmospheres that result from thermal decomposition are considered to be mixtures. In all other circumstances where a non-destructive test is used, the term test article should be used.

**Thoracic Fraction:** Fraction of aerosol that can reach the lung airways and the gas-exchange region. See also **Respirable fraction**, **Inhalable aerosol**.

**t<sub>95</sub>:** see **Equilibrium concentration**.

**Vapour:** The gaseous phase of a test article, including mixtures, which is normally in a liquid or solid state at ambient temperature and pressure. The vapour phase over a liquid is a diffusivity-dependent balance of evaporation and condensation. As a consequence of surface tension, vapour pressure is greater for small liquid droplets than for a plane surface (see Kelvin effect). See also **Evaporation**.

**Wall loss:** Deposition of particles in a sampler on surfaces other than those designed for particle collection (*e.g.*, chamber and tubing walls).

**Vapour saturation concentration:** For a vapour, the mass (m) and the molecular mass (M) of the evaporated liquid equilibrate as shown below. The approximate vapour saturation concentration can be estimated as follows:

$$C_{sat} = \frac{pM}{RT} \left( \frac{mg}{L} \right)$$

where p is the vapour pressure (atm) at the specified absolute temperature T (K), M is the molecular mass (mg), and R is the gas constant which is R = 0.082 (L atm)/(K Mol) or in SI units R = 8.314 J/(K Mol) where 1 L atm = 1.01328 • 10<sup>2</sup> J. J (Joule) is the unit of energy in N(Newton) • m. 1 Pa (Pascal) ≈ 1.0 J•L<sup>-1</sup>. The unit of Pa is N • m<sup>-2</sup>.

Temperature:  $T [^{\circ}\text{K}] = 273.15 + \text{degree Celsius}$

Pressure conversions:  $1 \text{ Pa} = 10^{-5} \text{ bar} = 0.987 \cdot 10^{-5} \text{ atm} = 0.0075 \text{ Torr}$ .

**Example calculation:**

The molecular mass of a test article is 100 g and its vapour pressure at 20 °C is 2 Pa.

$$C_{sat} = \frac{0.987 \times 10^{-5} \times 2 \times 100 \times 10^3}{0.082 \times (273.15 + 20)} \left( \frac{\text{atm} \times \text{mg} \times \text{K} \times \text{Mol}}{\text{Mol} \times \text{L} \times \text{atm} \times \text{K}} \right) = 0.082 \left( \frac{\text{mg}}{\text{L}} \right)$$

or in SI units:

$$C_{sat} = \frac{2 \times 10^{-3} \times 100 \times 10^3}{8.314 \times (273.15 + 20)} \left( \frac{\text{J} \times \text{mg} \times \text{K} \times \text{Mol}}{\text{Mol} \times \text{J} \times \text{K} \times \text{L}} \right) = 0.082 \left( \frac{\text{mg}}{\text{L}} \right)$$

**Whole-body chamber:** An inhalation chamber that exposes the whole animal. Especially for aerosols, this results not only in inhalation exposure, but also dermal exposure and oral exposure (via licking of the fur).

## APPENDIX II

### GHS CLASSIFICATION SYSTEM FOR ACUTE INHALATION (LC<sub>50</sub>)

In this system, substances can be allocated to one of the five toxicity categories based on acute toxicity by the inhalation route according to the numeric cut-off criteria shown below. Acute toxicity values are expressed as (approximate) LC<sub>50</sub> values or as Acute Toxicity Estimates (ATE). The concentrations to be used in limit tests are the upper bounds of Class 4 (20,000 ppm for gases, 20 mg/L for vapours, and 5 mg/L for aerosols) (11).

GHS Class	LC <sub>50</sub>		
	Gases (ppm) <sup>a</sup>	Vapours (mg/L)	Aerosols (dusts and mists) (mg/L)
<b>1</b>	≤ 100	≤ 0.5	≤ 0.05
<b>2</b>	> 100 and ≤ 500	> 0.5 and ≤ 2	> 0.05 and ≤ 0.5
<b>3</b>	> 500 and ≤ 2500	> 2 and ≤ 10	> 0.5 and ≤ 1
<b>4</b>	> 2500 and ≤ 20,000	> 10 and ≤ 20	> 1 and ≤ 5
<b>5</b>	> 20,000	> 20	> 5

a) The use of units of ppm for gases in the GHS Classification System leads to a disparity of classification between gases and vapours (which are in units of mg/L) even though both are gaseous. The disparity increases beyond the molecular weight of 122. For a molecular weight of 122, the conversion factor from ppm to mg/L is 0.005.

Note: For some substances or mixtures the test atmosphere will not just be a vapour but will consist of a concentration-dependent phase equilibrium of liquid and vapour phase.

### GHS Conversions from Acute Toxicity Range Values to Acute Toxicity Point Estimates

Conversion from experimentally obtained acute toxicity range values (or acute toxicity hazard classes) to acute toxicity point estimates for classification of **gases**.

Classification Class or Experimentally Obtained Acute Toxicity Risk Estimate (ppm)	Converted Acute Toxicity Point Estimate (ppm) <sup>a</sup>
0 < Class 1 ≤ 100	10
100 < Class 2 ≤ 500	100
500 < Class 3 ≤ 2500	700
2500 < Class 4 ≤ 20,000	4500
Class 5 > 20,000 <sup>a</sup>	See note b

Conversion from experimental obtained acute toxicity range values (or acute toxicity hazard classes) to acute toxicity point estimates for classification of **vapours**.

Classification Class or Experimentally Obtained Acute Toxicity Risk Estimate (mg/L)	Converted Acute Toxicity Point Estimate (mg/L) <sup>a</sup>
0 < Class 1 ≤ 0.5	0.05

$0.5 < \text{Class 2} \leq 2.0$	0.5
$2.0 < \text{Class 3} \leq 10.0$	3
$10.0 < \text{Class 4} \leq 20.0$	11
$\text{Class 5} > 20.0^a$	See note b

Conversion from experimental obtained acute toxicity range values (or acute toxicity hazard classes) to acute toxicity point estimates for classification of **aerosols** (dusts and mists).

Classification Class or Experimentally Obtained Acute Toxicity Risk Estimate (mg/L)	Converted Acute Toxicity Point Estimate (mg/L) <sup>a</sup>
$0 < \text{Class 1} \leq 0.05$	0.005
$0.05 < \text{Class 2} \leq 0.5$	0.05
$0.5 < \text{Class 3} \leq 1.0$	0.5
$1.0 < \text{Class 4} \leq 5.0$	1.5
$\text{Class 5} > 5.0^a$	See note b

<sup>a</sup> These values are designed to be used in the calculation of the ATE for classification of a mixture based on its components and do not represent test results. The values are conservatively set at the lower end of the range of Classes 1 and 2, and at a point approximately one tenth from the lower end of the range for Classes 3-5.

<sup>b</sup> From GHS (2007)(11) "...Criteria for Category 5 are intended to enable the identification of substances which are of relatively low acute toxicity hazard but which under certain circumstances may present a danger to vulnerable populations. These substances are anticipated to have an oral or dermal LD50 in the range of 2000-5000 mg/kg bodyweight and equivalent doses for inhalation. The specific criteria for Category 5 are:

(i) The substance is classified in this Category if reliable evidence is already available that indicates the LD50 (or LC50) to be in the range of Category 5 values or other animal studies or toxic effects in humans indicate a concern for human health of an acute nature.

(ii) The substance is classified in this Category, through extrapolation, estimation or measurement of data, if assignment to a more hazardous category is not warranted, and:

reliable information is available indicating significant toxic effects in humans;

any mortality is observed when tested up to Category 4 values by the oral, inhalation, or dermal routes; or

where expert judgement confirms significant clinical signs of toxicity, when tested up to Category 4 values, except for diarrhoea, piloerection or an ungroomed appearance; or

where expert judgement confirms reliable information indicating the potential for significant acute effects from other animal studies.

Recognizing the need to protect animal welfare, testing in animals in Category 5 ranges is discouraged and should only be considered when there is a strong likelihood that results of such a test would have a direct relevance for protecting human health"



# APPENDIX III

## COMPARISON OF TEST GUIDELINES

Study Design	TG 403 revised (2008) Traditional LC <sub>50</sub> study	TG 403 revised (2008) C×T study	TG 436 (2008)
<b>Exposure Methodology</b>			
Method	Traditional LC <sub>50</sub>	Concentration x time (C×T)	Acute Toxic Class method
Major endpoint	Mortality	Mortality	Mortality
Major objective	<ul style="list-style-type: none"> <li>• Concentration response for lethal and non-lethal endpoints (endpoints are system independent)</li> </ul>	<ul style="list-style-type: none"> <li>• Concentration response for lethal and non-lethal endpoints (endpoints are system independent).</li> <li>• Derivation of n in <math>C^n \times t</math></li> </ul>	<ul style="list-style-type: none"> <li>• Range estimate determination</li> </ul>
Use of data	<ul style="list-style-type: none"> <li>• Classification &amp; labelling by multiple systems including the GHS System.</li> <li>• Risk assessment.</li> <li>• Derivation of LC<sub>x</sub> values for one specific duration (usually 4 hours) for specific regulatory requirements (<i>e.g.</i>, emergency response, land-use planning).</li> <li>• Use of data for additional fixed exposure duration/day inhalation studies serving other regulatory demands (<i>e.g.</i>, inhalation mutagenicity studies, subacute and subchronic inhalation studies, inhalation developmental toxicity studies).</li> <li>• Clear appreciation of non-corrosive threshold concentrations as required by animal welfare regulations.</li> <li>• Detection of phase changes (<i>e.g.</i>, aerosol/vapor).</li> </ul>	<ul style="list-style-type: none"> <li>• Classification and labelling by multiple systems including the GHS System.</li> <li>• Risk assessment.</li> <li>• Derivation of LC<sub>x</sub> values for multiple durations for specific regulatory requirements (<i>e.g.</i>, emergency response, land-use planning).</li> <li>• Use of data for additional fixed exposure duration/day inhalation studies serving other regulatory demands (<i>e.g.</i>, inhalation mutagenicity studies, subacute and subchronic inhalation studies, inhalation developmental toxicity studies).</li> <li>• Clear appreciation of non-corrosive threshold concentrations as required by animal welfare regulations.</li> <li>• Detection of phase changes (<i>e.g.</i>, aerosol/vapor).</li> </ul>	<ul style="list-style-type: none"> <li>• Classification and labelling by the GHS System only (the fixed concentrations used in this Guideline are based on GHS cut-offs).</li> <li>• Not suitable for risk assessment.</li> </ul>
Mode of exposure	Nose-only or whole-body	Nose-only (whole-body chambers cannot be used)	Nose-only or whole-body
Concentrations tested	Variable—selected by the study director.	Variable—selected by the study director.	Gases : 100, 500, 2500, 20,000 ppm Vapours : 0.5, 2.0, 10.0, 20.0 mg/L Aerosols : 0.05, 0.5, 1.0, 5.0 mg/L
Atmosphere: concentration	Gases and vapours: ±10% Aerosols : ±20%	Gases and vapours: ±10% Aerosols : ±20%	Gases and vapours: ±10% Aerosols: ±20%

<b>Study Design</b>	<b>TG 403 revised (2008) Traditional LC<sub>50</sub> study</b>	<b>TG 403 revised (2008) C×T study</b>	<b>TG 436 (2008)</b>
variability			
Atmosphere: stability	Monitor continuously or hourly	Monitor continuously or hourly	Monitor continuously or hourly
Particle sizing (method)	At least twice during 4 hour exposure (cascade impactor)	At least twice during 4 hour exposure (cascade impactor)	At least twice during 4 hour exposure (cascade impactor)
Concentrations tested	Limit test: 1 Main study: At least 3	Limit test: 1 Main study: 4-5	1 or more
Exposure duration	Variable (generally 4 hours)	5 durations per concentration	4 hours
Particle size (aerosols)	MMAD: 1-4µm GSD: 1.5-3	MMAD: 1-4µm GSD: 1.5-3	MMAD: 1-4µm GSD: 1.5-3
Observation period	At least 14 days	At least 14 days	At least 14 days
Vehicle control group	Not generally required (historical data required if interactions cannot be excluded)	Not generally required (historical data required if interactions cannot be excluded)	Not generally required (historical data required if interactions cannot be excluded)
<b>Animals Tested</b>			
Limit test	3 ♂ and 3 ♀ (or 3 of the susceptible sex)	1-2 ♂ and 1-2 ♀ x 5 durations (or 2-4 of the susceptible sex) = 10-20 animals*	3 ♂ and 3 ♀ (or 6 of the susceptible sex)
Sighting study	≤ 3 ♂ and ≤ 3 ♀ (or ≤ 3 of the susceptible sex) per concentration	≤ 3 ♂ and ≤ 3 ♀ per concentration	0 (sighting studies are not used)
Main study	5 ♂ and 5 ♀ (or 5 of the susceptible sex) per concentration	10-20 animals (or 10-20 of the susceptible sex) x 5 durations per concentration*	3 ♂ and 3 ♀ (or 6 of the susceptible sex)
Total animals used in a non-limit study	If 3 concentrations are tested (typical): Both sexes = 30 Susceptible sex = 15	If 4 concentrations are tested (typical): Both sexes = 40-80 Susceptible sex = 40-80	If 1 concentration is tested: 6 If 2 concentrations are tested: 12 If 3 concentrations are tested: 18

\* Refer to paragraphs 54 and 55 regarding the number of animals to be used per C×T interval (18).

## APPENDIX IV

### PARTICLE SIZE DISTRIBUTION

To verify graphically that an aerosol is in fact unimodal and log-normally distributed, the normalized mass per stage ( $f_H'$ ) is evaluated as a histogram.  $\Delta \log D_p$  is equal to the difference  $\log D_{p+1} - \log D_p$ , whereas  $D_p$  is the lower cut-size limit and  $D_{p+1}$  the higher cut-size limit of the corresponding impactor stage. Calculate the histogram  $f_H'$  by this equation:

$$f_H' = N_f \times \frac{\text{mass / stage}}{\Delta \log D_p} \quad (1)$$

Calculate the log-normal mass distribution  $y'(D_{ae}) = N_f \times y(D_{ae})$  as a function of the aerodynamic diameter ( $D_{ae}$ ) using this equation:

$$y'(D_{ae}) = \exp \left[ -\frac{(\log D_{ae} - \log MMAD)^2}{2 \times \log^2 GSD} \right] \quad (2)$$

and use the normalization factor ( $N_f$ ):

$$N_f = \left( \frac{\Sigma \text{mass}}{\log GSD \times \sqrt{2\pi}} \right)^{-1} \quad (3)$$

An example calculation is provided in Table 1 and Fig. 2.

For non-modal particle size distributions other modes of evaluation may apply.

**Table 1:** Example table for cascade Impactor Analyses

N	Impactor Stage ( $\mu\text{m}$ - $\mu\text{m}$ )	Cut-Off Diameter ( $\mu\text{m}$ )	Mass/Stage (mg)	Relative Mass (%)	Cumulative Mass (%)
1	0.06 - 0.12	0.60	0.003	0.03	0.00
2	0.12 - 0.25	0.120	0.007	0.07	0.03
3	0.25 - 0.49	0.250	0.214	2.04	0.10
4	0.49 - 0.90	0.490	1.132	10.82	2.14
5	0.90 - 1.85	0.900	4.398	42.02	12.96
6	1.85 - 3.69	1.850	3.454	33.00	54.98
7	3.69 - 7.42	3.690	1.224	11.70	87.98
8	7.42 - 14.80	7.420	0.034	0.32	99.68
9	14.80 - 30.00	14.800	0.000	0.00	100.00

Mass Median Aerodynamic Diameter (MMAD): 1.66  $\mu\text{m}$   
Geometric standard deviation (GSD): 1.80

System: CASCADE-IMPACTOR  
Airflow: 5.85 L/min.  
Sampling time: 60.00 seconds  
Concentration (computed): 1789.06  $\text{mg}/\text{m}^3$

Respirability (percent < 1.0  $\mu\text{m}$ ):

-----  
Mass related: 19.7 %

Respirability (percent < 3.0  $\mu\text{m}$ ):

-----  
Mass related: 84.1 %

Respirability (percent < 5.0  $\mu\text{m}$ ):

-----  
Mass related: 96.9 %

**Figure 2:** Upper panel - plot of the percentage of mass less than the stated size (probability scale) *versus* aerodynamic particle size (log scale). Lower panel: Particle-size distribution  $h$  and histogram and log-normal distribution (equation 2).

